Peripheral blood mononuclear cell depletion for experimental human lung inflammation

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DECLARATION

This thesis is my own work. I carried out the studies described; the specific contributions of other individuals and members of my research group are clearly acknowledged.

This work has not been submitted in candidature for any other degree or professional qualification.

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ABSTRACT

Acute lung injury (ALI) affects a significant proportion of patients requiring critical care and is associated with high morbidity and mortality. Treatment is currently only supportive, with no pharmacological treatment yet shown to definitively improve outcome. There is evidence from murine models of ALI that monocytes play a key role in the development of the neutrophilic lung infiltration characteristic of ALI. Depletion of blood monocytes in mice given intra-tracheal lipopolysaccharide (LPS) significantly reduces pulmonary neutrophil influx, systemic neutrophilia and other markers of lung injury. In humans, monocyte-like cells have been documented in the bronchoalveolar lavage (BAL) fluid of patients with a variety of inflammatory lung conditions, including ALI. This thesis describes novel work performed in healthy human subjects to test whether, in an experimental model of human lung inflammation, depletion of circulating blood monocytes can ameliorate systemic and pulmonary inflammation.

LPS inhalation is an established method of modelling ALI in healthy human subjects as it safely and consistently induces mild and self-limiting systemic and pulmonary inflammation. A preliminary study in a group of 12 healthy subjects confirmed the safety and efficacy of LPS inhalation compared to saline placebo. LPS inhalation induced a marked blood neutrophilia together with a rise in body temperature and heart rate and elevated BAL neutrophil and pro-inflammatory cytokine concentrations. This study also used flow cytometry to confirm the presence of pulmonary monocyte-like cells (PMLCs) in BAL fluid, which, although distinct from blood monocytes, could be clearly divided into two separate sub-types according to CD14/CD16 expression. LPS inhalation caused a rise in the number of circulating classical monocytes in blood and an expansion in the $CD14^{++}CD16^{-}$ 'inducible' iPMLC subtype (reminiscent of classical blood monocytes), compared to the $CD14^{++}CD16^{+}$ 'resident' rPMLC subtype. This may represent transmigration of classical monocytes from blood across the pulmonary endothelium.

In humans, mononuclear cell (MNC) leukapheresis provides a readily available method of depleting circulating blood monocytes. A second preliminary study, performed in a separate group of 6 healthy subjects, demonstrated that leukapheresis of four total blood volumes could be safely employed to deplete large numbers of circulating blood monocytes. Active recruitment of monocytes into circulating blood during leukapheresis did, however, limit the reduction in total circulating blood monocyte counts. This study also investigated, for the first time, the potential pulmonary effects of leukapheresis. Despite a relative prominence of iPMLCs in BAL fluid after leukapheresis, there was no evidence of significant neutrophil influx or a clinically important pro-inflammatory effect in the alveolar space.

A randomised, double blind, placebo-controlled trial was then performed in a third group of 30 healthy human subjects who all inhaled LPS at baseline. There was no evidence that MNC leukapheresis (depletion group, n=15), compared to a sham procedure (sham group, n=15), attenuated the systemic and pulmonary inflammation induced by LPS inhalation, as measured by: blood neutrophil and plasma C-reactive protein (CRP) levels; by the neutrophil, protein and pro-inflammatory cytokine content of BAL fluid; and by [^{18}F]fluorodeoxyglucose positron emission tomography ([^{18}F]FDG PET)-derived measures of global lung inflammation. MNC leukapheresis temporarily prevented the LPS-induced rise in circulating classical monocytes and was also associated with a small reduction in the estimated numbers of MNCs in BAL fluid. It did not, however, appear to affect the LPS-induced expansion in the iPMLC subtype. Further characterisation of the PMLC subtypes by flow cytometry/sorting and cell culture demonstrated that the iPMLC subtype was more pro-inflammatory but less mature and with a lower proliferation potential than the rPMLC subtype.

In summary, this work did not support a role for circulating blood monocytes in the evolution of LPS-induced systemic or pulmonary neutrophilia in man. The rise in circulating levels of classical blood monocytes and the dramatic expansion of pro-inflammatory, immature iPMLCs in BAL fluid after LPS inhalation do, however, suggest that monocytes migrate to the lung and are to some extent involved in the pathogenesis of lung inflammation. Compared to murine methods of monocyte depletion, leukapheresis could not achieve such an extensive or sustained reduction in circulating blood monocyte counts, nor was it likely to have influenced other (specifically patrolling or splenic) monocyte pools. Future work in the drive to find treatments for ALI should therefore investigate the potential of pre-emptive leukapheresis or the efficacy and safety of other methods of human monocyte depletion in experimental lung inflammation.

ABBREVIATIONS

ACD-A	acid-citrate-dextrose solution A
ACE	angiotensin converting enzyme
AE	adverse event
AF	Alexa Fluor®
ALI	acute lung injury
ALT	alanine aminotransferase
AM	alveolar macrophage
ANCOVA	analysis of covariance
APC	allophycocyanin
ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
BCA	bicinchoninic acid
BP	blood pressure (prefix: d, diastolic; s, systolic)
BSA	bovine serum albumin
CBA	cytometric bead array
CC-16	Clara cell (16kD) protein, also known as uteroglobin
CCRE	Clinical Center Reference Endotoxin
CCR2	chemokine C-C motif receptor 2 (the receptor for MCP-1)
CD	cluster of differentiation
CD206	mannose receptor
CD62E	E-selectin
CD62L	L-selectin
CD71	transferrin receptor
$CI_{.95}$	95% confidence interval
COPD	chronic obstructive pulmonary disease
CPB	cardiopulmonary bypass
CRIC	Clinical Research Imaging Centre (University of Edinburgh)
CRP	C-reactive protein
CSU	Cell Separator Unit (South-East Scotland Blood Transfusion Service)
CT	computerised tomography
CXR	chest x-ray
CX3CR1	CX3C chemokine receptor 1, also known as the fractalkine receptor
DAMP	damage-associated molecular pattern
DC	dendritic cell
DLCO	diffusing capacity for carbon monoxide
DMSC	Data Monitoring and Safety Committee
DT(R)	diphtheria toxin (receptor)
E. coli	*Escherichia coli*
ECMO	extra-corporeal membrane oxygenation
EDTA	ethylene diamine tetra-acetic acid
eGFR	estimated glomerular filtration rate
ELF	epithelial lining fluid
ELISA	enzyme-linked immunosorbent assay
ENA-78	epithelial-derived neutrophil-activating peptide 78, also known as C-X-C motif chemokine 5 (CXCL5)
EVLP	ex-vivo lung perfusion
FBC	full blood count
FDG	fluorodeoxyglucose
FEV_1	forced expiratory volume in 1 second

FiO_2	fraction of inspired oxygen
FITC	fluorescein isothiocyanate
FSC-A	forward scatter-area
FVC	forced vital capacity
G(M)-CSF	granulocyte(-macrophage)-colony stimulating factor
GFP	green fluorescent protein
GLA	gamma-linolenic acid
GMF	geometric mean fluorescence
Gr-1	granulocyte differentiation antigen 1 (also known as Ly6G)
Hb	haemoglobin
HLA-DR	human leucocyte antigen-DR
HNE	human neutrophil elastase
HR	heart rate
ICAM	intercellular adhesion molecule
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
ILD	interstitial lung disease
IQR	interquartile range
ITU	intensive care unit
IV	intravenous
KGF	keratinocyte growth factor
K_i	influx constant (derived from a Patlak plot)
LAL	*Limulus* Amoebocyte Lysate
LBP	lipopolysaccharide-binding protein
LFA-1	lymphocyte function-associated antigen 1
LPS	lipopolysaccharide
LTA	lipoteichoic acid
LVRDS	large volume reservoir delivery system
Ly6C	lymphocyte antigen 6C
MAPK	mitogen-activated protein kinase
MBq	megabecquerel
MCP-1	monocyte chemotactic protein-1, also known as chemokine C-C motif ligand 2 (CCL2)
MIP-1α	macrophage inflammatory protein-1α, also known as chemokine C-C motif ligand 3 (CCL3)
MMAD	mass median aerodynamic diameter
MMP	matrix metalloproteinase
MNC	mononuclear cell
MPO	myeloperoxidase
MRI	magnetic resonance imaging
MSC	mesenchymal stem cell
MWU	Mann Whitney *U* statistical test
NAC	N-acetylcysteine
NF-κB	nuclear factor-kappa B
NM	neuromuscular
NO	nitric oxide
NS	non-significant
OD	optical density
OLV	one-lung ventilation
PAA	plasminogen activator activity
PAI-1	plasminogen activator inhibitor-1
PAMP	pathogen-associated molecular pattern

PBS	phosphate-buffered saline
PBSC	peripheral blood stem cell
PaO_2	partial pressure of oxygen in arterial blood
PE	phycoerythrin
PEEP	positive end-expiratory pressure
PerCP	peridinin-chlorophyll-protein complex
PET	positron emission tomography
PGE	prostaglandin E
PMLC	pulmonary monocyte-like cell (prefix: i, inducible; r, resident)
QMRI	Queen's Medical Research Institute (University of Edinburgh)
RA	rheumatoid arthritis
RAGE	receptor for advanced glycation end-products
RBC	red blood cell
RCT	randomised controlled trial
RFI	relative fluorescence intensity
rhAPC	recombinant human activated protein C
RIE	Royal Infirmary of Edinburgh
RML	right middle lobe (of the lung)
ROS	reactive oxygen species
RR	respiratory rate
SaO_2	oxygen saturation
SAE	serious adverse event
SD	standard deviation
SDF-1(α)	stromal cell-derived factor-1(α), also known as chemokine C-X-C motif ligand 12 (CXCL12)
SP-D	surfactant protein-D
SSC-A	side scatter-area
sTNFR	soluble tumour necrosis factor receptor
SUV	standardised uptake value
TAT	thrombin-antithrombin
TBV	total blood volume
tPA	tissue plasminogen activator
TF	tissue factor
T_h cell	T helper cell
TLR	toll-like receptor
TNFα	tumour necrosis factor alpha
TPR	tissue-to-plasma activity ratio
T_{reg} cell	regulatory T cell
VCAM	vascular cell adhesion molecule
vWF	von Willebrand factor
WBC	white blood cell
WTCRF	Wellcome Trust Clinical Research Facility (at the RIE)
25F9	anti-human mature macrophage marker

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CHAPTER 1: INTRODUCTION

1.1 OVERVIEW

Chapter 1 will explore the background to my thesis, explaining how my hypotheses were generated and how the studies I conducted aimed to answer these hypotheses.

I shall first consider the epidemiology, pathogenesis and consequences of acute lung injury (ALI). Next, I shall describe in detail the development and use of experimental models of human lung inflammation and the quest for effective treatments for ALI. The potential role of monocytes in lung inflammation will then be discussed, exploring the evidence available from murine models of lung injury and from inflammatory lung disease in humans. I will next outline murine studies of monocyte depletion in lung injury. I shall then describe the rationale for using leukapheresis to deplete circulating blood monocytes in humans.

Finally, I will outline my hypotheses and provide an overview of the studies carried out to test these hypotheses.

1.2 ACUTE LUNG INJURY

a) Definition and causes

The acute respiratory distress syndrome (ARDS) was first recognised in 1967 and reflects the more severe end of a spectrum of ALI. ALI is an inflammatory lung condition, defined as the acute development of persistent and refractory

hypoxaemia, in the presence of bilateral alveolar infiltrates on the chest x-ray (CXR), where there is no clinical evidence of a cardiogenic aetiology (raised left atrial pressure). ALI is characterised by a partial pressure of arterial oxygen (PaO_2) to fraction of inspired oxygen (FiO_2) ratio of ≤300mmHg (G R Bernard *et al.*, 1994). ARDS (severe ALI) is diagnosed when the PaO2 to FiO2 ratio is ≤200mmHg; although this is an arbitrary threshold, lower ratios are associated with higher mortality rates (Brun-Buisson *et al.*, 2004).

A relatively recent report by an expert panel aimed to update the consensus definition (Ferguson *et al.*, 2012; ARDS Definition Task Force *et al.*, 2012). The validity of this new 'Berlin' definition was tested on a large, international cohort of more than 4000 patients and aimed to improve reliability in relation to patient care and future research. The report defined ARDS (rather than ALI) as a PaO_2 to FiO_2 ratio ≤300mmHg in the presence of a positive end-expiratory pressure (PEEP) of ≥5 cm H_2O. It recommended stratifying patients into three categories of severity: mild ARDS (PaO2 to FiO2 ratio >200mmHg), moderate ARDS (PaO2 to FiO2 ratio >100mmHg) and severe ARDS (PaO2 to FiO2 ratio ≤100mmHg) (Ferguson *et al.*, 2012; ARDS Definition Task Force *et al.*, 2012). It was also agreed that a pulmonary artery wedge pressure ≤18mmHg does not fully exclude the presence of ARDS, in particular after large-volume fluid resuscitation (Wheeler and G R Bernard, 2007). It is as yet unclear whether this newer definition will be widely adopted. In this thesis, to maintain clarity, I shall use the term ALI to mean the entire spectrum of acute lung injury, including ARDS.

ALI affects a heterogeneous patient group; it can be induced directly by an intrinsic (pulmonary) cause such as pneumonia, gastric aspiration, inhalation injury including near-drowning or pulmonary contusion, or indirectly by a systemic cause, for example, sepsis, burns, extensive trauma, large-scale transfusion of blood products, pancreatitis, cardiopulmonary bypass and fat or amniotic fluid embolism (G R Bernard *et al.*, 1994; Wheeler and G R Bernard, 2007). Complex genetic, host and environmental factors are thought to explain why some patients with a typical predisposing event develop ALI and why some do not; similarly, genetic and environmental factors are thought to influence whether ALI resolves or progresses to cause multi-organ failure and death (Gao and Barnes, 2009; Glavan *et al.*, 2011).

b) Epidemiology of ALI

The worldwide incidence of ALI has been reported at anywhere between 2 and 75 cases per 100,000 person-years (Wheeler and G R Bernard, 2007). This wide variation in estimated incidence is thought to stem from differences in baseline population and patient identification and management. Approximately 190,000 cases occur annually in the U.S.A. (G R Bernard *et al.*, 1994; Rubenfeld *et al.*, 2005) and ALI affects around 7-8% of critically ill patients in the intensive care unit (ITU) setting in Europe and in Scotland (Brun-Buisson *et al.*, 2004; M Hughes *et al.*, 2003).

Mortality from ALI was originally reported at more than 50%; although this now appears to be declining, levels of morbidity and mortality remain

unacceptably high (Rubenfeld, 2007; Erickson *et al.*, 2009). Using the updated Berlin Definition, mortality has recently been estimated at 27% for mild ARDS, 32% for moderate ARDS and 45% for severe ARDS (ARDS Definition Task Force *et al.*, 2012). The median duration of mechanical ventilation also increases with the stage of severity in survivors of ARDS, from 5 days in mild cases, to 7 days in moderate cases and to 9 days in severe cases (ARDS Definition Task Force *et al.*, 2012).

Despite wide-ranging research, at present treatment of ALI essentially remains supportive, with no pharmacological therapy yet shown to reduce mortality. The high morbidity and mortality of ALI, plus significant use of hospital resources present ongoing drivers in the search for potential treatments. Furthermore, patients who survive ALI frequently display long-term functional consequences. Persistent impairment of pulmonary function, in particular carbon monoxide diffusion capacity, is often mild and asymptomatic. Instead, functional limitation is almost always due to neuromuscular or cognitive dysfunction (Herridge *et al.*, 2003; Rubenfeld, 2007).

c) Pathophysiology of ALI

ALI is a complex inflammatory state thought to represent an imbalance between pro- and anti-inflammatory mechanisms, such that an initially protective host immune response to an injurious stimulus evolves into an overwhelming, uncontrolled and ultimately harmful inflammatory cascade (Matthay and Zimmerman, 2005).

ALI is characterised by neutrophil infiltration of the pulmonary vasculature, interstitium and alveoli and reduced integrity of the barrier between the pulmonary endothelium and alveolar epithelium. The increased permeability of the 'blood-air barrier' results in the accumulation of protein-rich oedema fluid within the alveoli (Holter *et al.*, 1986; Matthay and Zimmerman, 2005). This is detectable by a steep rise in protein levels detectable in bronchoalveolar lavage (BAL) fluid. Respiratory failure develops due to reduced alveolar oxygenation, increased pulmonary dead space, reduced lung compliance and increased ventilation/perfusion mismatch (Wheeler and G R Bernard, 2007; Matthay *et al.*, 2012). Histological specimens demonstrate diffuse alveolar damage, with oedema, inflammation, haemorrhage and hyaline membrane formation due to fibrin deposition (Ware and Matthay, 2000; Matthay and Zimmerman, 2005).

The precise interactions of molecular and cellular mediators, pathways and mechanisms in ALI remain unknown. It is also unclear how much these differ depending upon the cause of ALI. Activation of the host's innate immune response is triggered locally or systemically through the detection of pathogen- or damage-associated molecular patterns (PAMPs and DAMPs) by pattern recognition receptors (Calfee and Matthay, 2010; Zhang *et al.*, 2010). Examples of pattern recognition receptors include toll-like receptors (TLRs), which are widely expressed by phagocytes and epithelial cells, and the receptor for advanced glycation end products (RAGE) (Di Candia *et al.*, 2012; Janssens and Beyaert, 2003).

The accumulation of neutrophils within the alveolar space appears to represent a key event in the majority of cases of ALI, although ALI can develop in neutropenic patients (Laufe *et al.*, 1986; Ognibene *et al.*, 1986), indicating the importance of other cell types including monocytes (whose role in ALI is discussed in detail later in this chapter), macrophages, platelets and lymphocytes. Higher levels of BAL neutrophils at an early stage are associated with greater mortality in ALI caused by both sepsis and trauma (Steinberg *et al.*, 1994) and neutrophil depletion appears beneficial in most animal models of ALI (Grommes and Soehnlein, 2011).

Activated neutrophils release a variety of harmful, cytotoxic substances that are crucial to microbial killing and host defence. Excessive activation of neutrophils can, however, cause tissue damage and, together with leucocyte migration itself, compromise the integrity of the endothelial-epithelial barrier. Reactive oxygen species (ROS) and cationic peptides, including defensins, are cytotoxic when released outwith the phagosome (Zemans *et al.*, 2009). Activated neutrophils release lactoferrin and myeloperoxidase (MPO, a lysozomal enzyme that catalyses the release of ROS) (Grommes and Soehnlein, 2011). Levels of proteinases, including human neutrophil elastase (HNE) and matrix metalloproteinases (MMPs) are raised in both plasma and BAL fluid of patients with ALI, and can indicate severity (Donnelly *et al.*, 1996; Zemans *et al.*, 2009). In addition to degradation of the extracellular matrix, MMPs play a role in neutrophil migration (Davey *et al.*, 2011).

A variety of chemotactic cytokines are involved in the attraction of leucocytes to the pulmonary vasculature adjacent to the site of inflammation. Neutrophil chemotaxis occurs in response to the release of interleukin (IL)-8 and other chemokines such as epithelial-derived neutrophil-activating peptide 78 (ENA-78) by AMs and epithelial cells (Grommes and Soehnlein, 2011; Soehnlein and Lindbom, 2010). Monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 alpha (MIP-1α), lactoferrin, stromal cell-derived factor-1 (SDF-1) and IL-8 are important mediators in monocyte chemotaxis (Gerszten *et al.*, 1999; Grommes and Soehnlein, 2011; Soehnlein and Lindbom, 2010). SDF-1 attracts lymphocytes to sites of inflammation and may also be involved in late neutrophil influx (Petty *et al.*, 2007).

Early release of the pro-inflammatory cytokines tumour necrosis factor-alpha (TNFα) and IL-1β is thought to initiate and augment the inflammatory process (Park *et al.*, 2001). IL-8 and MIP-1α also have pro-inflammatory effects. An imbalance between the levels of pro- and anti-inflammatory cytokines is thought to underpin the development of ALI (Ware and Matthay, 2000). For example, lower levels of anti-inflammatory cytokines, such as IL-10 and IL-1 receptor antagonist (IL-1ra) in BAL fluid are associated with poorer outcomes in patients with ALI (Donnelly *et al.*, 1996; Park *et al.*, 2001). Soluble TNF receptors (sTNFR) exert an anti-inflammatory effect by binding circulating TNFα (Grommes and Soehnlein, 2011). Some cytokines involved in the pathogenesis of ALI have mixed pro- and anti-inflammatory properties, for example, IL-6 and IL-12 (Park *et al.*, 2001).

Cytokine-mediated activation of pulmonary endothelial cells is thought to be key to the sequestration of leucocytes and has been the focus of much investigation. Although little is known about differences between leucocytes (in particular, neutrophils, monocytes and lymphocytes), their transendothelial migration into the pulmonary interstitium involves several steps (Imhof and Aurrand-Lions, 2004; Ley *et al.*, 2007).

Up-regulation of selectin (cell adhesion molecule) expression promotes initial 'capture' of leucocytes to the vascular endothelial wall, along which the leucocytes can then 'roll' (Ley *et al.*, 2007; Nourshargh *et al.*, 2010). L-selectin is expressed by most leucocytes and is believed to play an important role in leucocyte sequestration (Kuebler *et al.*, 2000); E-selectin and P-selectin are expressed by activated endothelial cells (Zemans *et al.*, 2009) and P-selectin is also expressed by platelets (Zarbock and Ley, 2009). Upregulation of other cell adhesion molecules, for example intercellular and vascular adhesion molecules (ICAM1 and VCAM1) by activated vascular endothelium encourages 'firm adhesion' of leucocytes through the binding of transmembrane receptors called integrins (Imhof and Aurrand-Lions, 2004; Ley *et al.*, 2007). Leucocytes then tend to 'crawl' to the site of transendothelial migration, which can occur by either a paracellular or transcellular route (Nourshargh *et al.*, 2010).

After passing through the endothelial layer, leucocytes then cross the basement membrane and pericyte sheath to enter the interstitium. Migration through the interstitium appears to be driven by the priming effect of prior transendothelial migration and rearrangement of the actin cytoskeleton within the cells (Ley *et*

al., 2007; Nourshargh *et al.*, 2010). Compared to transendothelial migration, transepithelial migration remains poorly delineated. Integrins are involved in leucocyte adherence to the basolateral epithelial surface. Leucocytes migrate across the epithelium in groups, by the paracellular route between type I and type II alveolar epithelial cells (Zemans *et al.*, 2009).

Plasma levels of von Willebrand factor (vWF) have been shown to act as a marker of endothelial cell activation and injury and higher levels are associated with poorer outcome in ALI (Ware *et al.*, 2004). Both production of surfactant and alveolar fluid clearance are adversely affected by damage to alveolar epithelial cells. Several biomarkers for alveolar epithelial damage have been discovered (McAuley, 2009). Surfactant protein-D (SP-D) is a large collagenous glycoprotein that acts as a marker of injury to type II alveolar epithelial cells; its levels in plasma correlate with severity of ALI, as does lower alveolar fluid clearance (Ware *et al.*, 2010). RAGE is strongly expressed by type I alveolar epithelial cells; BAL and plasma levels of soluble RAGE are higher in patients with ALI and baseline levels are linked with outcome (Uchida *et al.*, 2006; Calfee *et al.*, 2008). Clara cell secretory protein (CC-16) is mainly released by Clara cells within the terminal bronchioles of the lung (Hermans and A Bernard, 1999). Serum levels of CC-16 are a sensitive marker of increased epithelial permeability (Broeckaert and A Bernard, 2000; Determann *et al.*, 2009); however, higher levels of CC-16 in BAL fluid correlate with increased survival (Jorens *et al.*, 1995; Hermans and A Bernard, 1999).

ALI is also characterised by altered haemostasis and the formation of platelet-fibrin complexes within the alveolar space and pulmonary circulation (Ware *et al.*, 2007). Coagulation pathways become activated and there is higher expression of tissue factor (TF) and lower levels of anti-coagulant proteins (Günther *et al.*, 2000). For example, levels of protein C are reduced and this is associated with greater mortality (Ware *et al.*, 2007). There is also reduced fibrinolytic activity, with a rise in the levels of plasminogen activator inhibitor-1 (PAI-1), an anti-fibrinolytic protein released by epithelium, endothelium and fibroblasts within the lung, that again correlates with increased mortality (Günther *et al.*, 2000). Activation of coagulation pathways is thought to induce the release of pro-inflammatory cytokines and promote leucocyte transendothelial migration (Ware *et al.*, 2007; Schultz *et al.*, 2009).

Finally, there is also increasing interest in the mechanisms involved in the resolution of ALI, as these may be crucial to preventing the development of multi-organ failure and death or the progression of inflammation to fibrosing alveolitis (Matthay and Zimmerman, 2005). Neutrophil clearance and apoptosis may be important; phagocytosis of apoptotic neutrophils appears to make AMs express an anti-inflammatory phenotype (Savill *et al.*, 2002). The role of regulatory T (T_{reg}) cells is also under investigation (Venet *et al.*, 2009; Wang *et al.*, 2012). Improving alveolar oedema fluid clearance and re-establishing the integrity of the endothelial-epithelial barrier are vital steps in the resolution of lung injury; patients with better alveolar fluid clearance have reduced mortality and are ventilated for shorter periods (Ware and Matthay, 2001). Various

mediators, including MMPs, lipoxins and resolvins, appear to be involved in the process of alveolar epithelial repair (Davey *et al.*, 2011).

Further work is required to fully elucidate the pathophysiological mechanisms underlying ALI and to identify further molecular and cellular targets for treatment. The following section describes the avenues that have already been explored in the drive to improve the outcome from ALI.

d) Treatment of ALI

Table 1A lists the various interventions that have been investigated in patients with ALI. Despite extensive investigation, including several large multi-centre clinical trials carried out by The ARDS Network group, no pharmacological treatment has yet been shown to definitively reduce mortality from ALI (Thompson and G R Bernard, 2011). More supportive measures, in particular low tidal volume (lung protective) ventilation and now also prone positioning, have offered the most encouraging results to date in the search for effective treatments for ALI.

There are several potential interventions that are currently undergoing further investigation. The prophylactic use of β-agonists is currently being investigated in patients undergoing oesophagectomy (Perkins *et al.*, 2011). Large multi-centre RCTs are currently underway in both the UK and the U.S., investigating the use of statins in patients with ALI (McAuley *et al.*, 2012; Thompson and G R Bernard, 2011).

Table 1A: Interventions that have been investigated in patients with ALI

INTERVENTIONS	POTENTIAL MECHANISM OF ACTION	OUTCOME
PHARMACOLOGICAL MEASURES		
Surfactant	Low surfactant levels/function in ALI	A pooled analysis showed no evidence of benefit in adults [a]
Corticosteroids	Immunosuppressant action	No evidence of benefit in early ALI although may have early role in persistent ALI [a,b,c]
PGE1	Anti-inflammatory action	A pooled analysis showed no improvement in mortality [a]
NAC	Anti-oxidant	A pooled analysis showed no improvement in mortality [a]
Ketoconazole	Anti-inflammatory action; may also inhibit coagulation pathways	No evidence of a reduction in mortality [d,e] May have a prophylactic role in high-risk patients [f]
Lisofylline	Reduces circulating levels of oxidised free fatty acids	No evidence of improved outcome (trial stopped early due to futility) [g]
Inhaled NO	Endothelial-derived vasodilator	No reduction in mortality but sometimes used as a rescue therapy in severe refractory hypoxaemia (can improve oxygenation index) [h]
rhAPC	Altered haemostasis in ALI	No reduction in mortality or duration of ventilation (IV administration) [i] Nebulised rhAPC may have a role but larger studies are needed to confirm this [j,k,l]
β-agonists	Increasing alveolar fluid clearance and promoting endothelial/epithelial cell repair	Evidence of increased mortality with IV administration [m] No evidence of benefit with aerosolised β-agonists (trial stopped early due to futility) [n]
Statins	Inhibition of hydroxyl-methylglutaryl coenzyme A reductase	Small study showed no reduction in mortality (but improved oxygenation index, non-pulmonary organ dysfunction and BAL IL-8 concentrations) [o]

Table 1A continued...

SUPPORTIVE MEASURES		
Low tidal volume ventilation	Minimising further (ventilator-induced) lung injury	Clear reduction in mortality and duration of ventilation [p,q,r,s]
Increased PEEP	Improving alveolar recruitment	No evidence of additional benefit (beyond the use of low tidal volume ventilation) [r,s,t]
NM blockers	Facilitating lung-protective ventilation	Reduced duration of ventilation; possible reduction in mortality [u]
Prone positioning	Improving ventilation-perfusion matching	Recent evidence of a reduction in mortality and duration of ventilation [v]
High frequency oscillatory ventilation	Minimising further (ventilator-induced) lung injury	Conflicting results – no definite evidence of improved mortality [w,x]
Conservative fluid management	Reducing alveolar oedema	Reduction in the duration of ventilation and ITU stay, but no definite reduction in mortality [y,z]
DIETARY MEASURES		
Enteral omega-3 fatty acids, GLA & anti-oxidants	Reduction in pro-inflammatory arachidonic acid metabolites	Trend towards increased mortality (trial stopped early for futility) [aa]
Trophic (minimal) enteral feeding	Unknown	No reduction in mortality or duration of ventilation [bb]

Abbreviations: PGE1, prostaglandin E1; NAC, N-acetylcysteine; NO, nitric oxide; rhAPC, recombinant human activated Protein C; IV, intravenous; NM, neuromuscular; GLA, gamma-linolenic acid.

References: [a] (Adhikari *et al.*, 2004), [b] (Meduri *et al.*, 2008), [c] (Steinberg *et al.*, 2006), [d] (Thompson and G R Bernard, 2011), [e] (The ARDS Clinical Trials Network, 2000), [f] (Yu and Tomasa, 1993), [g] (Adhikari *et al.*, 2007), [h] (The ARDS Clinical Trials Network, 2002), [i] (Liu *et al.*, 2008), [j] (Dixon, Schultz, Smith, *et al.*, 2010), [k] (Dixon, Schultz, Hofstra, *et al.*, 2010), [l] (Tuinman *et al.*, 2012), [m] (F G Smith *et al.*, 2012), [n] (Matthay *et al.*, 2011), [o] (Craig *et al.*, 2011), [p] (Amato *et al.*, 1998), [q] (Brower *et al.*, 2000), [r] (Brower *et al.*, 2004), [s] (Meade *et al.*, 2008), [t] (Mercat *et al.*, 2008), [u] (Papazian *et al.*, 2010), [v] (Guerin *et al.*, 2013), [w] (Sud *et al.*, 2013), [x] (Young *et al.*, 2013), [y] (Rosenberg *et al.*, 2008), [z] (Wiedemann *et al.*, 2006), [aa] (Rice, 2011), [bb] (Rice *et al.*, 2012).

A randomised controlled trial (RCT) investigating the prophylactic use of aspirin in patients at high risk of developing ALI is also underway (Kor *et al.*, 2012). A preliminary study is investigating whether IV keratinocyte growth factor (KGF) can improve oxygenation (Cross *et al.*, 2013).

A large study of extra-corporeal membrane oxygenation (ECMO) in critically ill patients with severe acute respiratory failure demonstrated improved survival without disability at 6 months; further work is, however, required to confirm these results (Peek *et al.*, 2009). Finally, the use of stem cell therapy (for example, bone marrow-derived mesenchymal stem cells, MSCs) in ALI represents a new area of research that appears to offer much potential and is currently under investigation in a multi-centre phase II RCT (Matthay *et al.*, 2010). Stem cells release paracrine factors (including anti-inflammatory cytokines, growth factors and antimicrobial peptides) and may play an important role in cellular repair and restoration of endothelial/epithelial permeability (Hayes *et al.*, 2012).

The search for treatments that will improve the outcome from ALI has therefore, so far, been relatively unsuccessful. This is probably partly due to our relatively limited understanding of the complex pathophysiology of ALI. It is also difficult to identify patients early, before lung injury becomes established (Levitt and Matthay, 2010; Gajic *et al.*, 2011). A large amount of research into ALI has been performed using animal models, which are valuable but also have drawbacks; work using animal models of lung inflammation is summarised in the following section.

1.3 MODELLING LUNG INFLAMMATION IN ANIMALS

Ideas for potential new treatments for ALI often stem from clinical observations of patients in intensive care and/or from *in vitro* work in the laboratory, for example, studying cultured cell lines. Over the past two decades, a number of animal models of ALI have evolved that can be used to test hypotheses relating both to the pathogenesis of ALI and its treatment. For example, valuable strategies often involve inhibition of potential key pathways or specific mediators, or genetic manipulation to examine the role of specific genes and the proteins they express.

The most frequently employed animal models of ALI are listed in Table 1B with a brief summary of their advantages and disadvantages. However, none of these models induce all of the characteristic features of human ALI.

Various animal species have been used to study ALI, including mice, rats, rabbits, dogs, sheep, pigs and non-human primates. There are clearly multiple differences between humans and other species that, at least to some extent, explain the difficulty in translating promising new treatments from animal models into effective treatments in human patients with ALI. For example, in stark contrast to research in mice that indicates a beneficial effect of β-agonists in ALI, the human studies so far performed have not found any improvement in mortality or markers of severity (Perkins *et al.*, 2006). In general, the animals studied in such research tend to be young and otherwise healthy, compared to human patients who are often relatively old with multiple comorbid conditions.

Table 1B: Animal models of acute lung injury

MODEL	COMMENTS
ACID ASPIRATION	Marked difference from aspiration-induced human ALI Disrupts alveolar-capillary barrier; pulmonary neutrophil infiltrate; very reproducible effects
BLEOMYCIN	Not a relevant cause of human ALI Early effects are inflammatory, later effects are fibrotic; no hyaline membrane formation
BACTERIAL INSTILLATION	IV or intra-tracheal route; models sepsis-induced ALI Variable neutrophil infiltration; no hyaline membrane formation Minimal disruption of alveolar-capillary barrier
CAECAL LIGATION AND PUNCTURE	Models sepsis-induced ALI but requires complex surgery Disruption of alveolar-capillary barrier with some neutrophil infiltration Limited hyaline membrane formation
HYPEROXIA	Not, by itself, a cause of human ALI Causes late epithelial cell injury; neutrophil infiltrate not necessary; reproducible effects
INSTILLATION OF LPS	IV or intra-tracheal route Pulmonary neutrophil infiltrate; mild alveolar-capillary barrier changes; very reproducible effects
ISCHAEMIA-REPERFUSION INJURY	Can be a cause of human ALI; complex surgery may be required Mild pulmonary neutrophil infiltration/alveolar-capillary barrier disruption Effects potentiated by concomitant use of LPS
MECHANICAL VENTILATION WITH HIGH TIDAL VOLUMES	Not by itself a cause of human ALI Modelling ventilatory strategies has been successfully translated into clinical practice in human ALI * Synergistic effect when combined with another inflammatory stimulus
OLEIC ACID INSTILLATION	Disruption of alveolar-capillary barrier Not a common cause of human ALI but induces similar pathological features; reproducible effects
SALINE LAVAGE	Depletes surfactant (which is not a cause of ALI in adult human patients) Elicits physiological changes of ALI Milder pulmonary neutrophil infiltration/damage to alveolar-capillary barrier Often combined with another inflammatory stimulus (e.g. mechanical ventilation or LPS)

Abbreviation: LPS, lipopolysaccharide. * Reference: (Brower *et al.*, 2000).

The animals are also often genetically very similar to each other (Matute-Bello *et al.*, 2008). The extensive development of genetic manipulation in mice and the wide availability of murine-specific kits and reagents for laboratory use are two of the key advantages of studying lung injury in mice (Matute-Bello *et al.*, 2008). Their small size can, however, cause practical issues. Radiological assessment and pre-mortem blood and BAL sampling are challenging in small rodents (Matute-Bello *et al.*, 2008). Physiological monitoring is also technically challenging, especially considering the murine respiratory rate (RR) is around 250 to 300 breaths per minute (Matute-Bello *et al.*, 2011). Using larger animals that are genetically closer to humans is much more expensive and also raises more ethical considerations, particularly in relation to non-human primates (Bastarache and Blackwell, 2009).

There are also some key biological differences between animal species and humans, especially with regards to innate immunity and the lungs (Mestas and C Hughes, 2004). TLRs vary between species, as do monocyte subtypes (this is discussed in detail later) and chemotactic cytokines (for example, the murine homologues of human IL-8 are MIP-2 and keratinocyte chemoattractant) (Mestas and C Hughes, 2004; Matute-Bello *et al.*, 2008). There are also major differences in macrophage populations, with pulmonary intravascular macrophages present in some species such as sheep and pigs, but not in humans, primates, rodents or rabbits. Interestingly, pulmonary intravascular macrophages appear to facilitate the development of ALI following an injurious stimulus (Matute-Bello *et al.*, 2008). Other reported differences between mice

and humans include NO pathways of microbial killing (Matute-Bello *et al.*, 2008), responses to oxidative stress (Gharib *et al.*, 2010) and proportions of circulating neutrophils (Matute-Bello *et al.*, 2011). There are also some anatomical differences between humans and other animal species and different rates of alveolar fluid clearance (Proudfoot *et al.*, 2011; Ware, 2008).

The problem of inter-species variability can be overcome by using human models of lung inflammation, although animal models remain extremely useful when studying the underlying pathophysiology of lung inflammation and for the initial *in vivo* stage when testing potential treatments for ALI. It remains difficult to translate findings from animal models into patients, who represent a heterogeneous group. The next section illustrates the increasing use of human models of ALI with the aim of bridging the gap between research involving animal models and clinical studies in sick patients with ALI.

1.4 MODELLING LUNG INFLAMMATION IN HUMANS

a) Types of model

Human models of ALI vary from *in vivo* models, where certain surgical procedures in the clinical setting are associated with high subsequent rates of ALI, through experimental models in healthy human volunteers, to *ex vivo* models using non-transplantable donor lungs (Table 1C).

Table 1C: Human models of acute lung injury

MODEL	COMMENTS
ONE-LUNG VENTILATION (OLV) *	Used in thoracic anaesthesia e.g. during lung resection or oesophagectomy [a,b,c] Variable effects, ranging from subclinical alveolar neutrophil infiltrate to alveolar oedema with damage to alveolar-capillary barrier, epithelial cell necrosis, haemorrhage with fibrin formation [a] Pathogenesis thought to involve ischaemia-reperfusion injury and effects of hyperoxia [a,d]
CARDIO-PULMONARY BYPASS (CPB)	Activation of complement, endothelial cells and leucocytes causes systemic inflammatory response [d] Ischaemia-reperfusion injury may contribute to pathogenesis Widespread subclinical effects; induces fulminant ALI in around 2% of patients [d,e]
LPS CHALLENGE †	IV, inhaled or bronchoscopic instillation in healthy subjects Mild, frequently subclinical, pulmonary/systemic inflammation reminiscent of ALI Safe and reproducible effects
ISOLATED PERFUSED LUNG	Ex vivo model employing ventilation and perfusion of donor lungs unsuitable for transplantation [f] Usually combined with another insult e.g. LPS instillation Limited resource requiring technical expertise; does not reflect a multi-organ system Variability between patients, but contralateral lobe provides a within-subject control
EX VIVO LUNG PERFUSION (EVLP)	Ex vivo model, used in clinical practice to recondition lungs prior to transplantation [g] Extremely limited resource requiring technical expertise in a specialist centre Longer organ viability may enable study of later stages of ALI

* OLV is associated with the subsequent development of ALI in 5% of patients undergoing lobectomy, 8% of patients undergoing pneumonectomy and over 20% of patients undergoing oesophagectomy.

† LPS challenge is discussed in much greater detail on Pages 37-59.

References: [a] (Jordan *et al.*, 2000), [b] (Proudfoot *et al.*, 2011), [c] (Tandon *et al.*, 2001), [d] (Clark, 2006), [e] (Asimakopoulos *et al.*, 1999), [f] (J W Lee *et al.*, 2009), [g] (Cypel *et al.*, 2011).

Phase II trials can be performed in high-risk patient groups, for example patients undergoing one-lung ventilation (OLV) or cardiopulmonary bypass (CPB), to establish the safety and efficacy of potential treatments and preventative strategies for ALI. However, the majority of patients develop ALI for other reasons. The development of LPS challenge as a model of early lung inflammation in healthy subjects has lead to increasing numbers of phase II trials in healthy subjects, as a prelude to phase III clinical trials in patients with ALI or the high-risk patient groups mentioned above.

The next sections will describe the discovery of LPS and the rationale for its use in modelling lung inflammation, plus more details on the types of LPS challenge available and their respective advantages and disadvantages. I will also report the results of previous research using LPS challenge to model human ALI.

b) The discovery of LPS and its inflammatory effects

LPS is purified bacterial endotoxin, which forms part of the outer leaflet of the outer membrane of Gram-negative bacteria. Endotoxin was discovered more than 100 years ago by Pfeiffer, who demonstrated that administering lysates of heat-inactivated *Vibrio cholerae* to laboratory animals still induced shock (endotoxin is heat-stable) (Beutler and Rietschel, 2003). Later, administration of endotoxin itself was shown to induce pyrexia and leucocytosis (Bayston and Cohen, 1990; Beutler and Rietschel, 2003). Further work was performed to optimise the extraction and purification of LPS from endotoxin and the chemical nature of LPS was eventually characterised by Westphal and Luderitz in the mid

20th century (Bayston and Cohen, 1990). LPS consists of an O-specific chain (a water-soluble polysaccharide component), which is linked by core oligosaccharide to Lipid A (the glycophospholipid component). The O-specific chain confers its unique antigenic properties, which vary between species and determine LPS serotype (e.g. *Escherichia (E.) coli* 026:B6). Lipid A is responsible for the biological activity of LPS (Bayston and Cohen, 1990).

LPS acts as a PAMP and the recognition of its presence by the innate immune system has been extensively investigated. TLR-4 is the key transmembrane receptor for LPS, forms a receptor complex with CD14 and is expressed by macrophages and epithelial cells within the alveolar space (Beutler and Rietschel, 2003). LPS binding to this receptor complex is dependent upon the presence of LPS-binding protein (LBP) and MD-2 (also known as lymphocyte antigen 96) (Beutler and Rietschel, 2003). LPS signalling to induce down-stream effects such as cytokine production is mediated by adaptor proteins (mainly MyD88) and occurs via the activation of the transcription factor nuclear factor-kappa B (NF-κB) and protein kinases including mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinases (Janssens and Beyaert, 2003).

Endotoxin is shed by Gram-negative bacteria and is therefore found widely in the environment, including in the nasal and oral passages of animals and humans and on plant, animal and soil surfaces (Radon, 2006). Endotoxin is found in high concentrations in some occupational environments; much of the early research on endotoxin and LPS was undertaken to investigate

occupational lung disease caused by organic dust inhalation, for example in relation to livestock and grain handling, textile production and waste processing (Radon, 2006). It was noted that workers exposed to cotton dust could develop transient 'flu-like symptoms, including pyrexia, cough, myalgia and tachycardia (Thorn, 2001). Tolerance does develop, with a reduction in symptoms upon repeated exposure and their reappearance after a period of no exposure, hence why this phenomenon is sometimes termed 'Monday Fever' (Pernis *et al.*, 1961). The first description of experimental inhalation of purified endotoxin in humans demonstrated that this could elicit symptoms identical to those observed after cotton dust inhalation (Pernis *et al.*, 1961).

Much of the early literature describes studies performed using un-purified endotoxin or cotton-dust extract, often concentrating on the airways effects in individuals with atopy or chronic lung disease (Thorn, 2001). In more recent years, there have been many studies investigating the inflammatory effects of purified LPS in healthy subjects (as well as in patients with atopy and chronic lung disease). LPS challenge can be either systemic, by IV injection, or directly to the lungs by inhalation or by segmental instillation using a fibreoptic bronchoscope.

c) Intravenous LPS

IV LPS mainly induces systemic effects and is thus more useful as a model of sepsis than specifically of lung injury. Most studies have used Clinical Reference Centre Endotoxin (CCRE, which is LPS from *E. coli* 0113). It has been given,

according to subject weight, in a dose of 1-4 ng/kg (Boujoukos *et al.*, 1993; Cannon *et al.*, 1990; Copeland *et al.*, 2005; Draisma *et al.*, 2009; Franco *et al.*, 2000; Kuhns *et al.*, 1995; Martich *et al.*, 1991; Rodrick *et al.*, 1992; Suffredini *et al.*, 1999; van Deventer *et al.*, 1990). Interestingly, sensitivity to IV LPS provides yet another example of differences between species, with mice requiring a much higher dose relative to body weight (about 250 times greater than humans) to achieve a similar cytokine response (Copeland *et al.*, 2005).

The IV administration of LPS induces pyrexia (which is dose-dependent and peaks around 3 to 5 hours), tachycardia, myalgia and a small fall in mean arterial pressure, all of which resolve by 24 hours (Granowitz *et al.*, 1993; Kuhns *et al.*, 1995; Suffredini *et al.*, 1999; Copeland *et al.*, 2005; Schinkel *et al.*, 2005). There is a rapid rise in the plasma concentrations of C-reactive protein (CRP) and several pro-inflammatory cytokines, including TNFα (peaking at 1.5 hours), IL-1β (peaking at 2 to 3 hours), IL-6 and IL-8 (which peak at 2 to 4 hours) (Richardson *et al.*, 1989; Cannon *et al.*, 1990; Martich *et al.*, 1991; Granowitz *et al.*, 1993; Kuhns *et al.*, 1995; Suffredini *et al.*, 1999; Copeland *et al.*, 2005; Draisma *et al.*, 2009). This is mirrored by rises in the plasma levels of anti-inflammatory mediators including IL-10, IL-1ra and sTNFR, which peak slightly later, at 3 to 6 hours (Spinas *et al.*, 1992; Granowitz *et al.*, 1993; Kuhns *et al.*, 1995; Copeland *et al.*, 2005; Draisma *et al.*, 2009).

Increased levels of plasma vWF (a marker of endothelial cell activation) are detectable as early as 1.5 hours after the administration of IV LPS, and peak around 6 hours (van Deventer *et al.*, 1990; Draisma *et al.*, 2009). Plasma levels

of soluble (s)E-selectin (shed by activated endothelial cells) also peak around 6 hours (Kuhns *et al.*, 1995).

The leucocyte response to IV LPS is characterised by a brief but clear fall in circulating neutrophil numbers, followed by a rapid rise, with numbers peaking around 4-6 hours (Richardson *et al.*, 1989; P D Smith *et al.*, 1994; Kuhns *et al.*, 1995; Suffredini *et al.*, 1999; Copeland *et al.*, 2005). Plasma granulocyte colony-stimulating factor (G-CSF) levels also rise by 4 hours, indicating neutrophil release from bone marrow and/or marginating pools (Kuhns *et al.*, 1995). IV LPS administration causes a fall in the circulating numbers of lymphocytes and monocytes (visible between 1 and 4 hours) (Richardson *et al.*, 1989; Suffredini *et al.*, 1999; Copeland *et al.*, 2005).

IV LPS also causes a rise in the levels of plasma HNE and lactoferrin, a marker of neutrophil degranulation (Kuhns *et al.*, 1995; Suffredini *et al.*, 1999; Draisma *et al.*, 2009). There are also increased plasma levels of sL-selectin, which is shed by activated neutrophils (Solomkin *et al.*, 1994; Kuhns *et al.*, 1995; van der Poll *et al.*, 1997).

IV administration of LPS has characteristic effects on coagulation. Initially, there is increased tissue-plasminogen activator (t-PA) activity and higher levels of plasmin-α2-antiplasmin complexes and D-dimers, indicating that fibrinolysis is transiently enhanced (Pajkrt *et al.*, 1997; Suffredini *et al.*, 1989; van der Poll *et al.*, 1997). This is followed by suppression of fibrinolysis, with increased PAI-1 concentrations (Pajkrt *et al.*, 1997; Suffredini *et al.*, 1989; van der Poll *et al.*,

1997; van Deventer *et al.*, 1990), and activation of coagulation, with a peak in prothrombin fragments, TF mRNA expression and thrombin-antithrombin (TAT) complexes in plasma by 3 hours (van Deventer *et al.*, 1990; van der Poll *et al.*, 1997; Franco *et al.*, 2000).

Although the initial neutropenia suggests circulating neutrophils rapidly become marginated after IV LPS, there is only minimal neutrophil transmigration into the alveolar space by 6 hours, with no clear rise in total BAL cell or differential neutrophil counts (Boujoukos *et al.*, 1993; P D Smith *et al.*, 1994; Suffredini *et al.*, 1999). Systemic LPS does, however, induce a minor impairment in pulmonary gas exchange and a subtle increase in lung epithelial permeability (Suffredini *et al.*, 1992). Furthermore, AMs retrieved 6 hours after systemic LPS administration are primed, releasing greater concentrations of TNFα and IL-1β when stimulated *in vitro* (P D Smith *et al.*, 1994).

d) Inhalation of LPS

LPS inhalation has been employed in more than 50 human studies, involving more than 1000 subjects, over the past few decades. Many of these studies have been performed in healthy subjects (Table 1D); however, some studies have recruited patients. There has, for example, been significant interest in the relevance of low-dose LPS in allergic sensitisation; many studies have now been performed in atopic and asthmatic patients, using comparatively much lower doses of LPS or incremental dosing protocols (Kitz *et al.*, 2006; Kline *et al.*, 1999; Michel *et al.*, 1989; Sundy *et al.*, 2006). Interestingly, work in mouse models has

suggested that low-level exposure to inhaled LPS primes T helper 2 (T_h2) cells, causing allergic sensitisation, compared to inhalation of higher concentrations of LPS, which leads to T_h1 responses including release of pro-inflammatory cytokines (Eisenbarth *et al.*, 2002). This adds credence to the hygiene hypothesis, whereby lower exposure to environmental endotoxin in childhood has been linked to a greater risk of atopic conditions (Strachan, 1989). Indeed, lower doses of inhaled LPS also appear to induce T_h2-type responses rather than neutrophilic inflammation in humans (Alexis *et al.*, 2004). Tolerance to repeated doses of LPS also appears to be linked to lower-dose LPS inhalation (Loh, 2005).

There appears to be a threshold dose of inhaled LPS, of around 30-40μg, for inducing clinical symptoms alongside a neutrophilic inflammatory response (Thorn, 2001). Most studies employing LPS inhalation as a model of acute lung inflammation have therefore used doses of at least 50μg (Table 1D). The effects of inhaling this dose of LPS have been measured by symptoms, spirometry (to assess airways effects), peripheral blood samples (to gauge systemic effects) and by transfer factor for carbon monoxide (DLCO), induced sputum and BAL (to assess pulmonary effects).

Inhalation of higher doses of LPS induces significant pyrexia in the majority of subjects, which may become noticeable as early as 4 hours and tends to peak between 8 to 14 hours post-challenge (Clapp *et al.*, 1993; Michel *et al.*, 2001; Maris, De Vos, Bresser, *et al.*, 2005; Loh *et al.*, 2006; Sundblad *et al.*, 2009).

Table 1D: Studies involving moderate- or high-dose LPS challenge directly to the lungs in healthy human subjects

AUTHOR	SUBJECTS	TYPE/DOSE OF LPS	ADMINISTRATION	DESIGN AND MEASURED OUTCOMES
(Pernis *et al.*, 1961)	n=3	*E.coli* 15-60 μg	Inhalation	Spirometry
(Cavagna *et al.*, 1969)	n=10	*E.coli* 40-80 μg	Inhalation	Spirometry, blood samples
(Herbert *et al.*, 1992)	n=8	*E.coli* 026:B6 100 μg dissolved in water vs. placebo	Inhalation by jet nebuliser (Pari Boy)	Crossover design, 2-week washout; spirometry, transfer factor, blood
(Clapp *et al.*, 1993)	n=7	*E.coli* O111:B4 30-60 μg vs. saline placebo, corn/dust extracts	Inhalation by dosimeter nebuliser (DeVilbiss)	Crossover design, 10-day washout; spirometry, blood samples, nasal lavage
(Michel *et al.*, 1995)	n=8	*E.coli* 026:B6 20 μg vs. saline placebo	Inhalation by dosimeter nebuliser (Mefar MB3)	Blinded design; spirometry, blood samples
(Jagielo, 1996)	n=14	*E.coli* O111:B4 30-60 μg vs. corn dust extract	Inhalation by dosimeter nebuliser (DeVilbiss)	3-week washout between challenges; spirometry, BAL (4h)
(Michel *et al.*, 1997)	n=9	*E.coli* 026:B6 L2654 0.5, 5 then 50 μg vs saline placebo	Inhalation by dosimeter nebuliser (Mefar MB3)	Blinded design, 1-week washout Spirometry, blood samples, induced sputum (6h)
(Wesselius *et al.*, 1997)	n=14	*E.coli* O111:B4 30 μg vs controls, smokers	Inhalation by nebuliser (Respirgard II)	Spirometry, blood samples, BAL (1.5 or 4h)
(Thorn and Rylander, 1998)	n=21	*E.coli* 026:B6 40 μg	Inhalation by jet nebuliser (Pari Boy)	Spirometry, blood, induced sputum (24h)
(Nightingale *et al.*, 1998)	n=11	*E.coli* 60 μg vs saline placebo	Inhalation by dosimeter nebuliser (Mefar MB3)	Randomised, double-blind, crossover, 4-week washout; spirometry, induced sputum (0, 6 and 24h)
(Michel *et al.*, 2001)	n=15	*E.coli* 026:B6 L2654 0.5, 5 then 50 μg vs saline placebo	Inhalation by dosimeter nebuliser (Mefar MB3)	Blinded design, one-week washout between challenges; spirometry, airways responsiveness, blood, induced sputum (6h)

Table 1D continued...

AUTHOR	SUBJECTS	TYPE/DOSE OF LPS	ADMINISTRATION	DESIGN AND MEASURED OUTCOMES
(O'Grady *et al.*, 2001)	n=34	*E.coli* O:113 (CCRE) 1-4 ng/kg in water vs saline placebo	Segmental instillation	Randomised to RML/lingula (for LPS/placebo); blood, BAL (2, 6, 24 or 48h)
(Michel *et al.*, 2003)	n=116	*E.coli* 026:B6 L2654 20 μg	Inhalation by dosimeter nebuliser (Mefar MB3)	Blood samples
(Nick, 2004)	n=16	*E.coli* O111:B4 (CCRE) 4ng/kg vs saline placebo	Segmental instillation	Randomised to RML/lingula (for LPS/placebo); randomised, double-blind, placebo-controlled study of rhAPC; blood samples, BAL (16h)
(Wallin *et al.*, 2004)	n=15	*E.coli* 026:B6 50μg vs saline placebo	Inhalation by jet nebuliser (Pari-Boy)	Double-blind, double-dummy crossover study of salmeterol versus placebo pre-treatment, 3-week washout; spirometry, BAL and endobronchial biopsies (3h)
(Roos-Engstrand *et al.*, 2005)	n=15	*E.coli* 026:B6 50μg vs saline placebo	Inhalation by jet nebuliser (Pari-Boy)	Two challenges (saline, then LPS), 3-week washout; endobronchial biopsies and BAL (3h).
(Maris, De Vos, Dessing, *et al.*, 2005)	n=32	*E.coli* 026:B6 100 μg vs saline placebo	Inhalation by LVRDS	Randomised (blinded) to salmeterol or saline placebo pre-treatment, then to LPS or saline placebo; BAL (6h)
(Maris, De Vos, Bresser, *et al.*, 2005)	n=6	*E.coli* 026:B6 50 μg vs saline placebo	Inhalation by LVRDS	Blinded design, crossover with 4-week washout; spirometry, BAL (6h)
(Michel *et al.*, 2005)	n=15	*E.coli* 026:B6 L2654 50 μg	Inhalation by dosimeter nebuliser (Mefar B3)	Methlyprednisolone or placebo pre-treatment; spirometry, blood
(Chen *et al.*, 2006)	n=18	*E.coli* O:113 (CCRE) in sterile water; 1, 2 or 4 ng/kg	Segmental instillation	n=6 for each dosing group; blood samples, [^{18}F]FDG PET (0 and 24h), BAL (29h)

Table 1D continued...

AUTHOR	SUBJECTS	TYPE/DOSE OF LPS	ADMINISTRATION	DESIGN AND MEASURED OUTCOMES
(Coldren *et al.*, 2006)	n=19	*E.coli* O:113 (CCRE) 4ng/kg vs saline placebo	Segmental instillation	Randomised to RML/lingula (for LPS/placebo); blood, BAL (16h)
(Abraham *et al.*, 2006)	n=15	*E.coli* O:113 (CCRE) 4ng/kg vs saline placebo	Segmental instillation	Randomised to RML/lingula (for LPS/placebo); blood, BAL (16h)
(Loh *et al.*, 2006)	n=18	*E.coli* 026:B6 15μg (n=10), 50 μg (n=8) vs saline placebo	Inhalation by dosimeter nebuliser (Mefar MB3)	Three separate challenges (placebo, LPS, placebo), 1-week washout; symptoms, blood, induced sputum (6h)
(Michel *et al.*, 2007)	n=18	*E.coli* 026:B6 L2654 50 μg	Inhalation by dosimeter nebuliser (Mefar MB3)	Randomised to pre-treatment with PGE4 inhibitor, prednisolone or saline placebo; double-blind/double-dummy/crossover, 8-day washout; blood, induced sputum (6h)
(Hoogerwerf *et al.*, 2008)	n=8	*E.coli* (CCRE) vs saline placebo and LTA	Segmental instillation	Randomised to RML/lingula (for LPS/placebo); blood, BAL (6h)
(LeVan *et al.*, 2008)	n=88	*E.coli* 026:B6 L2654 20 μg	Inhalation by dosimeter nebuliser (Mefar MB3)	Blood samples
(Hohlfeld *et al.*, 2008)	n=43	*E.coli* (CCRE) 4ng/kg vs saline placebo	Segmental instillation	LPS to RML, placebo to lingula; double-blind design, randomised to pre-treatment with PGE4 inhibitor or placebo; BAL (24h)
(Fouassier *et al.*, 2009)	n=12	*E.coli* 026:B6 L2654 50 μg	Inhalation by dosimeter nebuliser (Mefar MB3)	Serial blood samples
(Sundblad *et al.*, 2009)	n=12	*E.coli* O111:B4 53.4 μg vs pig-barn dust	Inhalation by dosimeter nebuliser (Spira)	Crossover study, 3-week washout Symptoms, spirometry, methacholine challenge, exhaled NO, induced sputum
(Shyamsundar *et al.*, 2009)	n=30	*E.coli* 026:B6 L2654 50 μg	Inhalation by dosimeter nebuliser (Spira)	Randomised (double-blind) to 40/80mg simvastatin vs placebo pre-treatment; blood samples, BAL (6h)

Table 1D continued...

AUTHOR	SUBJECTS	TYPE/DOSE OF LPS	ADMINISTRATION	DESIGN AND MEASURED OUTCOMES
(Chen *et al.*, 2009)	n=22	*E.coli* 0:113 (CCRE) 4ng/kg	Segmental instillation	Randomised (double-blind) to lovastatin (n=8), rhAPC (n=7) or placebo (n=7) pre-treatment; spirometry, blood samples, [^{18}F]FDG PET (0 and 24h), BAL (29h)
(Glader *et al.*, 2010)	n=12	*E.coli* O:113 (CCRE) 4ng/kg vs saline placebo	Segmental instillation	Randomised to RML/lingula (for LPS/placebo); BAL (12 or 24h)
(Frankenberger *et al.*, 2011)	n=4	*Salmonella abortus equi* 20 μg	Inhalation by nebuliser (AKITA)	Induced sputum (24h)
(Shyamsundar *et al.*, 2010)	n=36	*E.coli* 026:B6 L2654 50 μg	Inhalation by dosimeter nebuliser (Spira)	Randomised (double-blind) to KGF or placebo pre-treatment; BAL (6h)
(Korsgren *et al.*, 2011)	n=19	*E.coli* 026:B6 L2654 5 and 50 μg vs saline placebo	Inhalation by dosimeter nebuliser (Spira)	Three separate challenges, 2-week washout; spirometry, blood samples, induced sputum (4, 24 and 48h)

This table excludes studies of patients with asthma, atopy or chronic lung disease and excludes studies of only low-dose (≤15μg) inhaled LPS. LPS was dissolved in saline, unless otherwise specified. L2654 denotes LPS that has been gamma-irradiated and purified by gel-filtration chromatography (Sigma, Gillingham, UK).

Abbreviations: RML, right middle lobe, LVRDS, large-volume reservoir delivery system; [^{18}F]FDG PET, [^{18}F]fluorodeoxyglucose positron emission tomography; LTA, lipoteichoic acid.

A proportion of subjects report transient associated 'flu-like symptoms, including headache, malaise and myalgia, whose prevalence correlates to the dose of LPS inhaled (Nightingale *et al.*, 1998; Thorn and Rylander, 1998; Michel *et al.*, 2001; Loh *et al.*, 2006; Sundblad *et al.*, 2009; Korsgren *et al.*, 2011). LPS inhalation may lead to a small rise in heart rate (HR) but does not alter blood pressure (BP) or oxygen saturations (SaO_2) (Maris, De Vos, Bresser, *et al.*, 2005; Loh *et al.*, 2006; Shyamsundar *et al.*, 2009).

Chest tightness and cough are less frequently reported and may indicate mild LPS-induced bronchospasm (Jagielo, 1996). Although inhaled LPS can cause a significant (if only temporary) fall in the forced expiratory volume in 1 second (FEV_1), there is conflicting evidence as to frequency, with several studies reporting no significant change in spirometry (Michel *et al.*, 1995; Wesselius *et al.*, 1997; Nightingale *et al.*, 1998; Wallin *et al.*, 2004; Maris, De Vos, Bresser, *et al.*, 2005; Michel *et al.*, 2005; Loh *et al.*, 2006; Shyamsundar *et al.*, 2009). The reason probably partly relates to the difference between statistical significance and clinical significance (a clinically-significant fall in FEV_1 is usually defined as a $\geq$10% fall from baseline) (Sundblad *et al.*, 2009; Korsgren *et al.*, 2011). Secondly, any fall in FEV_1 probably occurs relatively early, peaking within one hour (Cavagna *et al.*, 1969; Clapp *et al.*, 1993; Michel *et al.*, 2001), and may therefore be missed if the subject is asymptomatic.

LPS inhalation consistently leads to a sharp rise in circulating white blood cells (WBCs) that is dose-related (Clapp *et al.*, 1993; Michel *et al.*, 2003; Loh *et al.*, 2006). This is due to an increase in the number of blood neutrophils, which

becomes evident by 4 hours and peaks around 8 hours post-inhalation (Herbert *et al.*, 1992; Michel *et al.*, 1995; Michel *et al.*, 2007; Korsgren *et al.*, 2011). WBC and neutrophil counts return to baseline levels between 24 and 48 hours post-challenge (Thorn and Rylander, 1998). LPS inhalation leads to even earlier activation of blood neutrophils (detectable by 2 hours); by 24 hours, plasma MPO is significantly raised (Thorn and Rylander, 1998).

LPS inhalation leads to elevated plasma levels of several pro-inflammatory cytokines, including TNFα, IL-1β and IL-6, which return to baseline by 24 hours (Jagielo, 1996; Wesselius *et al.*, 1997; Fouassier *et al.*, 2009; Korsgren *et al.*, 2011). Plasma CRP shows a marked rise, peaking between 24 and 48 hours (Michel *et al.*, 1995; Michel *et al.*, 2001; Michel *et al.*, 2003; Michel *et al.*, 2007; Korsgren *et al.*, 2011). Plasma LBP and sE-selectin also peak at 24 hours (Michel *et al.*, 2001; Michel *et al.*, 2003; Michel *et al.*, 2007). Plasma CC-16 levels are elevated at 6-8 hours, returning to baseline by 24 hours (Michel *et al.*, 2005).

In terms of local (pulmonary) effects, one study reported an early fall in DLCO between 2-4 hours after LPS inhalation (Herbert *et al.*, 1992). In induced sputum, LPS inhalation causes a significant rise in total cell numbers and a steep rise in both neutrophil content and absolute neutrophil numbers (Nightingale *et al.*, 1998; Loh *et al.*, 2006; Michel *et al.*, 2007; Sundblad *et al.*, 2009). The number of lymphocytes in induced sputum may also rise to a degree (Thorn and Rylander, 1998; Michel *et al.*, 2001). Along with sputum neutrophilia, there is a rise in sputum MPO and HNE concentrations (Thorn and Rylander, 1998; Michel *et al.*, 2001; Korsgren *et al.*, 2011), together with a rise in sputum TNFα, IL-8

and MMP-9 levels (Nightingale *et al.*, 1998; Michel *et al.*, 2001; Michel *et al.*, 2007; Korsgren *et al.*, 2011).

LPS inhalation causes a rise in total cell numbers in BAL fluid, which is apparent as early as 1.5 hours (Wesselius *et al.*, 1997). There is a steep rise in both neutrophil content and absolute neutrophil numbers in BAL by 4-6 hours (Jagielo, 1996; Wesselius *et al.*, 1997; Wallin *et al.*, 2004; Maris, De Vos, Dessing, *et al.*, 2005; Maris, De Vos, Bresser, *et al.*, 2005). Although the proportion of AMs in BAL falls (due to elevated neutrophil content), absolute AM numbers remain stable and may even rise (Wesselius *et al.*, 1997; Wallin *et al.*, 2004; Maris, De Vos, Dessing, *et al.*, 2005; Maris, De Vos, Bresser, *et al.*, 2005). Total protein levels in BAL fluid may show a small rise a few hours after LPS inhalation (Wesselius *et al.*, 1997). Along with a rise in neutrophil numbers, LPS inhalation increases BAL levels of MPO, HNE and S100A12 (the latter is released by activated neutrophils and acts as a ligand for RAGE) (Maris, De Vos, Bresser, *et al.*, 2005; Wittkowski *et al.*, 2007). There are early rises in the BAL levels of pro-inflammatory cytokines including TNFα, IL-1β, IL-6 and IL-8, detectable as early as 1.5 hours after LPS inhalation (Jagielo, 1996; Wesselius *et al.*, 1997; Maris, De Vos, Dessing, *et al.*, 2005; Maris, De Vos, Bresser, *et al.*, 2005; Roos-Engstrand *et al.*, 2005). Conversely, levels of IL-10 and IL-12p70 frequently remain undetectable (Maris, De Vos, Dessing, *et al.*, 2005; Maris, De Vos, Bresser, *et al.*, 2005). Levels of chemotactic cytokines, including ENA-78, MIP-1α and MIP-1β, are elevated in BAL after LPS inhalation (Maris, De Vos, Dessing, *et al.*, 2005). Tissue obtained by endobronchial biopsy demonstrates higher expression of

phosphorylated p39 MAPK after LPS inhalation, which is thought to lead to up-regulated inflammatory cytokine expression (Roos-Engstrand *et al.*, 2005). BAL fluid obtained after LPS inhalation induces much greater nuclear translocation of NF-κB in stimulated monocyte-derived macrophages (Shyamsundar *et al.*, 2009).

Of particular note, LPS inhalation activates coagulation pathways within the alveolar space, as demonstrated by a rise in the levels of TAT complexes, soluble TF and soluble thrombomodulin and a reduction in the levels of protein C and APC in BAL fluid (Maris, De Vos, Bresser, *et al.*, 2005; Maris *et al.*, 2007). Alveolar fibrinolysis is inhibited by LPS inhalation, with reduced levels of plasminogen activator activity (PAA) and increased levels of PAI-1 present in BAL fluid (Maris, De Vos, Dessing, *et al.*, 2005; Maris *et al.*, 2007). There is, however, no evidence that inhaled LPS affects systemic coagulation pathways (Fouassier *et al.*, 2009).

e) Segmental LPS challenge

A few studies have used an alterative approach to LPS inhalation, by directly instilling LPS into a single lung segment using fibre-optic bronchoscopy (Table 1C). The first study to describe segmental LPS challenge established the safety and efficacy of a 4ng/kg dose of CCRE and analysed the time-course of the inflammatory response by performing subsequent BAL at either 2, 6, 24 or 48 hours (O'Grady *et al.*, 2001). Most subsequent studies have also employed a dose of 4ng/kg and have used saline instillation into a segment of the

contralateral lung as a within-subject control. The effects of segmental challenge have been measured using symptoms, spirometry, blood sampling, BAL and also by [^{18}F]FDG PET imaging, as an alternative means of assessing the pulmonary effects of LPS challenge.

As with LPS inhalation, segmental challenge is associated with significant pyrexia by 8 hours (O'Grady *et al.*, 2001; Chen *et al.*, 2006; Hoogerwerf *et al.*, 2008; Chen *et al.*, 2009). Otherwise, the most frequently reported symptoms are headache, myalgia and also cough (particularly at the time of bronchoscopy) (O'Grady *et al.*, 2001; Chen *et al.*, 2009). With regards to clinical signs, mean arterial pressure and HR dip to a nadir at about 4 hours (O'Grady *et al.*, 2001; Chen *et al.*, 2006). There is also some evidence that segmental LPS challenge causes a small but significant fall in SaO_2 or PaO_2 (O'Grady *et al.*, 2001; Chen *et al.*, 2006). FEV_1 tends to fall to a small degree after LPS challenge; although this varies between subjects, any decline in FEV_1 is only temporary and usually asymptomatic (Chen *et al.*, 2006; Chen *et al.*, 2009).

As with inhaled LPS, segmental LPS challenge causes a rise in peripheral blood leucocytes, with an approximately two-fold rise in circulating blood neutrophil counts (O'Grady *et al.*, 2001; Hoogerwerf *et al.*, 2008). Segmental LPS challenge also causes a rise in the plasma levels of G-CSF, IL-1ra, IL-6 and CRP (O'Grady *et al.*, 2001; Hoogerwerf *et al.*, 2008).

In BAL fluid, segmental LPS challenge causes a marked rise (at least three-fold) in absolute cell counts by 6 hours, peaking at 24 hours (O'Grady *et al.*, 2001;

Chen *et al.*, 2006; Hoogerwerf *et al.*, 2008; Glader *et al.*, 2010). As with LPS inhalation, there is a dramatic rise in the absolute number of neutrophils present in BAL between 6 and 24 hours (O'Grady *et al.*, 2001; Coldren *et al.*, 2006; Hohlfeld *et al.*, 2008; Hoogerwerf *et al.*, 2008; Glader *et al.*, 2010). The absolute numbers of AMs/monocytes and lymphocytes also probably rise after segmental LPS challenge, albeit to a much lesser extent and at a later stage (peaking at 24-48 hours or later) (O'Grady *et al.*, 2001; Hohlfeld *et al.*, 2008; Glader *et al.*, 2010).

Levels of MPO and HNE are elevated in BAL fluid after segmental LPS challenge (O'Grady *et al.*, 2001; Hoogerwerf *et al.*, 2008). Neutrophils retrieved from BAL fluid demonstrate reduced spontaneous apoptosis as well as greater release of superoxide anions (Coldren *et al.*, 2006). Furthermore, there is a correlation between the neutrophil count in BAL fluid and the level of NF-κB activation in circulating blood neutrophils (Abraham *et al.*, 2006).

There is a clearer rise in total protein content of BAL fluid after segmental LPS challenge than that described after LPS inhalation (O'Grady *et al.*, 2001; Nick, 2004). TNFα is detectable in BAL fluid between 2 and 16 hours, peaking at around 6 hours (O'Grady *et al.*, 2001; Nick, 2004; Hohlfeld *et al.*, 2008; Hoogerwerf *et al.*, 2008). This is accompanied by a more persistent rise in the BAL concentrations of sTNFR1 and sTNFR2 (O'Grady *et al.*, 2001; Hoogerwerf *et al.*, 2008). Levels of IL-6, IL-8 and MMP-9 are also elevated in BAL fluid after segmental LPS challenge (O'Grady *et al.*, 2001; Nick, 2004; Abraham *et al.*, 2006; Hohlfeld *et al.*, 2008; Hoogerwerf *et al.*, 2008). Small rises in the BAL

concentrations of IL-1β and IL-1ra can be detected, but (as with LPS inhalation) levels of IL-10 and IL-12p70 remain undetectable after segmental LPS challenge (O'Grady *et al.*, 2001; Hoogerwerf *et al.*, 2008).

BAL fluid concentrations of G-CSF, ENA-78, MCP-1, MIP-1α and MIP-1β in BAL fluid are elevated by 6 hours after segmental LPS challenge and normalise by 24 hours (O'Grady *et al.*, 2001; Nick, 2004; Hohlfeld *et al.*, 2008; Hoogerwerf *et al.*, 2008). The level of sL-selectin in BAL fluid rises and remains high even at 48 hours after LPS challenge (O'Grady *et al.*, 2001).

AMs exposed to *in vivo* LPS challenge demonstrate enhanced mRNA expression for pro-inflammatory and chemotactic cytokines (including IL-1β, IL-6, IL-8, MCP-1 and TNFα) and, after culturing *in vitro*, display increased spontaneous release of IL-1β, IL-6 and IL-8, but not IL-10, IL-12p70 or TNFα (Hoogerwerf *et al.*, 2008; Hoogerwerf *et al.*, 2010).

As with inhalation, segmental LPS challenge causes activation of alveolar coagulation and suppression of fibrinolysis, with raised BAL levels of TAT complexes, soluble TF and thrombomodulin, D-dimer and PAI-1 and reduced levels of antithrombin, PAA, protein C and APC (Van Der Poll *et al.*, 2005; Hoogerwerf *et al.*, 2009).

Studies using PET-derived markers of pulmonary inflammation have demonstrated a dose-related rise in the rate of [^{18}F]FDG uptake from LPS-challenged lung segments when compared to control (saline-challenged) segments (Chen *et al.*, 2006; Chen *et al.*, 2009). Interestingly, the rate of

[^{18}F]FDG uptake demonstrated only a weak correlation with the neutrophil content of BAL after segmental LPS challenge (Chen *et al.*, 2006).

f) Advantages and limitations of LPS models

LPS models of lung inflammation clearly offer a controlled, reliable and safe method of activating the innate immune response in healthy subjects. The effects of LPS challenge are acceptable, reproducible and reflect many of the pulmonary and systemic changes seen in ALI. Furthermore, they spontaneously resolve without any known long-term consequences.

LPS challenge does, however, have a number of limitations. It is performed rapidly and the time-course over which lung inflammation evolves does not therefore reflect the 'real life' scenario. Patients can develop ALI due to pulmonary or non-pulmonary causes; in this regard, IV LPS does not model a pulmonary aetiology and LPS inhalation or segmental challenge do not model systemic causes of ALI. Safety is a key concern: the degree of lung inflammation induced in human LPS models is by necessity much milder than that induced in murine models of lung 'injury'. Idiosyncratic reactions, although rare, have been reported in the literature, for example one subject developed a widespread rash after LPS inhalation and another reported mouth ulcers as a side effect (Pernis *et al.*, 1961; Loh *et al.*, 2006).

Subject variability represents another limitation. The LPS challenge studies described above have used healthy subjects between the ages of 18-50, whereas patients are often older, with other health problems. Gender may also influence

results; one study described a greater systemic response to IV LPS in female compared to male subjects (Van Eijk *et al.*, 2007), although other studies have demonstrated little or no difference (Kuhns *et al.*, 1995; Coyle *et al.*, 2006). *In vitro* studies using peripheral blood have documented wide inter-subject variability in the production of inflammatory cytokines (Park, 2002). Some variability may be due to genetic differences; for example, single nucleotide polymorphisms of the CD14 and TLR genes have been shown to influence the inflammatory response to inhaled LPS (Michel *et al.*, 2003; LeVan *et al.*, 2008).

There have been substantial variations in the methodology of LPS inhalation studies in the past; factors that may alter the effective delivered dose and subject response to it include the type of LPS, its solvent, the type of nebuliser and the inhalation technique itself. Most LPS inhalation studies have used LPS from *E.coli* (Table 1D). Animal studies indicate that LPS from different Gram-negative bacteria have similar biological activity (Luchi and D C Morrison, 2000); using LPS from a single batch minimises any differences that might occur in biological activity. LPS is more soluble in water than in saline; however, most studies have used the latter, as inhalation of distilled water has been reported to cause both bronchospasm (thus potentially altering inhaled distribution) and a peripheral blood neutrophilia (Herbert *et al.*, 1992).

The type of nebuliser and the technique of inhalation probably have the greatest influence upon the dose of LPS actually delivered to the lower airways and, in particular, to the alveoli. Continuous nebulisation over 15 to 20 minutes (for example, by large volume reservoir delivery systems) has now largely been

replaced by the use of inhalation-triggered dosimeter-nebulisers and a standardised 5-breath technique similar to that used in methacholine challenge (Popa, 2001). Important considerations when using dosimeter-nebulisers include driving pressures, nebulisation onset and duration, using nose-clips and closing additional flow valves/vents to prevent variable air in-flow (Ryan *et al.*, 1981; Häkkinen *et al.*, 1999; Jõgi *et al.*, 1999; Popa, 2001).

Different dosimeter models produce different particles sizes, for example, the particle mass median aerodynamic diameter (MMAD) is estimated at 3.4-4μm for the Mefar MB3, compared to 3.5μm for the DeVilbiss 646 and 1.6μm for the Spira Elektro dosimeter-nebuliser (Jõgi *et al.*, 1999; Nieminen *et al.*, 1987). Alveolar deposition of inhaled particles is increased when particle MMAD is <5μm, and smaller MMADs (albeit >1μm) may potentiate this further (Häkkinen *et al.*, 1999; Brand *et al.*, 2000; Heyder, 2004).

There is evidence that moderate, controlled inspiratory flow rates (for example, up to 0.5 L/second) reduce extra-thoracic (upper airway) particle deposition and reduce overall deposition variability, possibly by reducing turbulence (Häkkinen *et al.*, 1999; Brand *et al.*, 2000; Heyder, 2004). Conversely, more rapid inhalation has been demonstrated to reduce distal particle deposition in the lung (Ryan *et al.*, 1981). Depth of inspiration is also a factor; breathing to full inspiratory capacity (as opposed to tidal breathing) is thought to minimise inhalation-induced bronchospasm (Jõgi *et al.*, 1999; Kapsali *et al.*, 2000; Cockcroft, 2005). This may to some extent explain why inhalation to maximal inspiratory capacity improves alveolar particle deposition (Ryan *et al.*, 1981;

Brand *et al.*, 2000; Heyder, 2004; Möller *et al.*, 2008). Furthermore, breath-holding for 5 seconds at full inspiration is thought to improve gravitational deposition of particles within the alveoli (Heyder, 2004).

It has been recommended that dosimeter-nebulisers should be calibrated every 20 uses, using the desired amount of sterile saline and weighing scales accurate to 0.5mg (Popa, 2001). Estimations of dosing should be performed in triplicate, using 10 slow inhalations, from functional residual capacity to maximal inspiratory capacity, with a 5-second breath-hold each time (Popa, 2001). It has been noted that both exhalation to the room (rather than re-breathing into the nebuliser mouthpiece) and using triggered inspiration (rather than manual activation) are important to avoid underestimation of dose during calibration (Cockcroft, 2005; Davis *et al.*, 2004; Todd *et al.*, 2005).

LPS models also have limitations in terms of design and also in the measurement of effects. Bronchoscopy to obtain BAL samples is clearly a more invasive procedure than obtaining induced sputum, but offers distinct advantages when modelling ALI. BAL samples are more likely to reflect the alveolar compartment, whereas induced sputum samples are more representative of the proximal bronchial tree (Lensmar *et al.*, 1998; Alexis *et al.*, 2001). As such, induced sputum samples tend to have lower numbers of total cells and macrophages and greater numbers of neutrophils, when compared to BAL samples (Lensmar *et al.*, 1998; Antoniou *et al.*, 2005). Furthermore, macrophages from induced sputum samples display greater activation,

oxidative burst and phagocytic activity than alveolar macrophages from BAL samples (Lensmar *et al.*, 1998; Alexis *et al.*, 2000).

As well as being invasive, bronchoscopy and BAL techniques often vary between studies and sample dilution can vary even when a standardised technique is used (Haslam and Baughman, 1999). Methods of adjusting for sample dilution also sometimes lack accuracy (Baughman, 1997; Walters and Gardiner, 1991). Furthermore, BAL fluid is not representative of pulmonary inflammation in its entirety, as BAL is only performed in one segment of one lobe; it also does not sample cells attached to the alveolar epithelium or those within the interstitium. Compared to BAL, [^{18}F]FDG PET scanning provides a better measure of 'whole' lung inflammation, in that it can record data from all lobes of both lungs (Harris and Schuster, 2007). [^{18}F]FDG activity is also reflective of both alveolar and interstitial neutrophils. It has its own limitations, however, in that it is not specific to neutrophils and does not enable characterisation of cellular phenotype or detailed function.

Provision of a control in LPS inhalation studies involves either a separate placebo challenge (in the same subject) with an appropriate washout period, or a separate control group where different subjects inhale placebo. Segmental LPS challenge has the advantage that a simultaneous within-subject control (saline challenge) can be performed using a segment within a contralateral lobe. It does, however, necessitate two bronchoscopy procedures (if later BAL is performed). It is also unclear whether saline or LPS instillation at baseline bronchoscopy can have subtle, indirect effects on subsequent BAL samples.

g) Interventional studies employing LPS challenge

Table 1E summarises the studies that have been performed using LPS challenge in healthy human subjects to test potential interventions in ALI, many of which have then been followed by phase III RCTs in patients with ALI (as discussed under 'Treatment of ALI', Page 26).

Table 1E: Intervention studies employing LPS inhalation or segmental challenge

TREATMENT	STUDY DETAILS	RESULTS
β-agonists	(Wallin *et al.*, 2004), n=15, double-blind design 3 weeks of salmeterol vs placebo LPS inhalation; BAL at 3h	No significant reduction in LPS-induced rise in BAL leucocytes, neutrophils or total protein
	(Maris, De Vos, Dessing, *et al.*, 2005; Maris *et al.*, 2007), n=32, single-blind design Single salmeterol vs placebo inhalation LPS/placebo inhalation at 30 minutes; BAL at 6h	Significant reduction in LPS-induced rise in BAL neutrophil levels and in BAL MPO and TNFα levels; non-significant reduction in LPS-induced rise in BAL IL-8 levels No reduction in LPS-induced activation of coagulation; significant enhancement of pulmonary fibrinolysis
Corticosteroids	(Michel *et al.*, 2005), n=15, double-blind design 6-days of methylprednisolone vs placebo LPS/placebo inhalation; serial plasma samples	Significant reduction in LPS-induced rise in plasma CC-16 levels
	(Michel *et al.*, 2007), n=16, double-blind design 6 days prednisolone or placebo LPS inhalation; induced sputum at 6h	No reduction in LPS-induced rise in sputum leucocytes or neutrophil content; no reduction in LPS-induced rise in blood neutrophil counts; no reduction in LPS-induced rise in plasma sE-selectin or LBP; significant reduction in LPS-induced rise in plasma CRP
Phosphodiesterase-4 inhibitors	(Michel *et al.*, 2007), n=16, double-blind design 6 days cilomilast vs placebo LPS inhalation, induced sputum at 6h	No reduction in LPS-induced rise in sputum leucocytes or neutrophil content; no reduction in LPS-induced rise in blood neutrophil counts; no reduction in LPS-induced rise in plasma sE-selectin or LBP; trend towards reduction in LPS-induced rise in plasma CRP levels
	(Hohlfeld *et al.*, 2008), n=37, double-blind design 28 days roflumilast or placebo Segmental LPS & saline control challenge; BAL at 24h	Significant reduction in LPS-induced rise in BAL leucocyte and neutrophil counts; no effect upon pro-inflammatory cytokines in BAL

Table 1E continued...

TREATMENT	STUDY DETAILS	RESULTS
rhAPC	(Nick, 2004; Van Der Poll *et al.*, 2005), n=16, double-blind design 16-hour rhAPC or placebo infusion Segmental LPS & saline control challenge at 2 hours; BAL at 18h	Significant reduction in BAL leucocyte and neutrophil numbers; no change in blood neutrophil counts No change in total protein, IL-6, IL-8 or MCP-1 levels in BAL; No significant change in kinase phosphorylation or neutrophil cell death; significant reduction in IL-8-induced neutrophil chemotaxis Significant attenuation of LPS-induced procoagulant effect; no overall effect on LPS-induced inhibition of fibrinolysis
	(Chen *et al.*, 2009), n=12, double-blind design 29 hours rhAPC or placebo Segmental LPS challenge at 2 hours; PET at 24h, BAL at 29h	Trend towards reduction in LPS-induced rise in [^{18}F]FDG uptake and reduction in leucocyte and neutrophil counts in BAL
Recombinant human KGF	(Shyamsundar *et al.*, 2010), n=36, double-blind design 3 days palifermin or placebo LPS inhalation; BAL at 6h	No reduction in LPS-induced rise in BAL leucocytes, neutrophils, total protein, TNFα, IL-8 or MCP-1
Statins	(Shyamsundar *et al.*, 2009), n=30, double-blind design 4 days simvastatin or placebo LPS inhalation; BAL at 6h	Significant reduction in LPS-induced BAL neutrophilia, MPO, TNFα, MMP-7 and MMP-9 and trend towards reduction in IL-1β and MMP-2 levels; significant reduction in LPS-induced rise in plasma CRP; no reduction in BAL total protein levels
	(Chen *et al.*, 2009), n=12, double-blind design 2 days lovastatin Segmental LPS challenge; PET at 24h, BAL at 29h	Significant reduction in LPS-induced rise in [^{18}F]FDG uptake; trend towards reduced leucocyte and neutrophil counts in BAL

1.5 THE ROLE OF MONOCYTES IN ACUTE LUNG INJURY

The previous section described the background and rationale for using LPS challenge to model early ALI in healthy subjects and thus provide the opportunity to test potential interventions. This section will present evidence for the role of monocytes in ALI, describe work performed in animal models to test the effects of monocyte depletion and will then go on to discuss monocyte depletion in the human setting.

a) Circulating and marginating monocytes

Monocytes are involved in both the innate and adaptive immune response, with multiple functions including release of cytokines and inflammatory mediators, phagocytosis and antigen presentation (Auffray *et al.*, 2009). Monocytes are derived from macrophage and dendritic cell progenitors within the bone marrow; these can also develop into classical and plasmacytoid dendritic cells (DCs) (Auffray *et al.*, 2009; Geissmann *et al.*, 2010; Robbins and Swirski, 2010).

Upon maturation, monocytes are released from the bone marrow to circulate in peripheral blood for up to 3 days (Gordon and Taylor, 2005; Whitelaw, 1972). In mice, the release of monocytes from bone marrow appears to be CCR2-dependent (Serbina and Pamer, 2006; Tsou *et al.*, 2007). Circulating monocytes do not appear to be capable of proliferation; for example, they do not express a key protein called Ki-67 (Geissmann *et al.*, 2003; Van Furth *et al.*, 1979; Whitelaw, 1972). Monocytes are believed to migrate into the tissues where they evolve to become macrophages or 'inflammatory' DCs, a process that can be

demonstrated *in vitro* by culture with IL-4 and GM-CSF (Gordon and Taylor, 2005; Geissmann *et al.*, 2008).

Migration into tissues involves an intermediate step whereby monocytes become attached to vascular endothelium (a process known as 'margination'). In animals, the marginating pool of monocytes is actually larger than the circulating pool (Van Furth and Sluiter, 1986; Ohgami *et al.*, 1991). Furthermore, using fluorescent staining and intravital microscopy, one group has demonstrated the presence of a large population of splenic monocytes in mice (Swirski *et al.*, 2009). These monocytes reside in the subcapsular red pulp (rather than the splenic vasculature) and are more numerous than their counterparts circulating in blood. It has not yet been confirmed whether a similar splenic monocyte population exists in humans.

b) Murine monocyte subsets

The study of murine monocytes was accelerated by the use of mice with green fluorescent protein (GFP) 'knocked-in' to one of the fractalkine receptor alleles (CX3C chemokine receptor 1, or CX3CR1) (Geissmann *et al.*, 2003; Gordon and Taylor, 2005; Robbins and Swirski, 2010). Cells of the monocyte lineage in these $Cx_3cr1^{gfp/+}$ mice demonstrate green fluorescence when examined by microscopy (Geissmann *et al.*, 2010). The intensity of GFP expression by murine monocytes was noted to be either high or intermediate, indicating the existence of two separate monocyte subsets that are present in equal proportions (Geissmann *et*

al., 2003; Sunderkötter *et al.*, 2004). Table 1F lists the main phenotypic differences in cell surface marker expression between the two subsets.

Table 1F: Key differences in cell surface marker expression by the two major murine monocyte subsets

PHENOTYPE	'INFLAMMATORY' (~50%)	'RESIDENT' (~50%)
CD115	+	+
CX3CR1	Moderate (GFP)	High (GFP)
Ly6C	High	Low
Gr-1	+	-
CCR2	+	-
L-selectin	+	-
LFA-1 integrin	Low	High
CD43	Low	High

Abbreviations: Ly6C, lymphocyte antigen 6C; Gr-1, granulocyte differentiation antigen 1 (also known as Ly6G); LFA-1 integrin, lymphocyte function-associated antigen-1.
Data derived from (Auffray *et al.*, 2009; Geissmann *et al.*, 2003; Geissmann *et al.*, 2008; Gordon and Taylor, 2005; Grage-Griebenow *et al.*, 2001; Ziegler-Heitbrock *et al.*, 2010; Robbins and Swirski, 2010; Tacke and Randolph, 2006; Sunderkötter *et al.*, 2004; Weber *et al.*, 2000; Wong *et al.*, 2011).

The 'inflammatory' subset is Ly6C^{high} and consists of larger monocytes (average diameter of 10-14μm), with a shorter half-life, that migrate to sites of inflammation (Geissmann *et al.*, 2010).

The second 'resident' subtype is Ly6C^{low} and consists of smaller monocytes (average diameter 8-12μm) that were initially described as present in 'resting' (non-inflamed) tissues (Geissmann *et al.*, 2010). This subtype does, however, demonstrate rapid recruitment to inflamed tissues, with marked but transient release of TNFα (Auffray *et al.*, 2009). One of the main functions of resident

murine monocytes is thought to be in tissue repair and angiogenesis (Geissmann *et al.*, 2010).

There is conflicting evidence over whether these two murine monocyte subsets represent a spectrum of maturation. Bone marrow monocytes appear to express high levels of Ly6C, which fall over time (at least in *ex vivo* culture) (Sunderkötter *et al.*, 2004). Although *in vivo* monocyte depletion is followed by sequential repopulation (first by Ly6C^{high}, followed by Ly6C^{low} monocytes) (Sunderkötter *et al.*, 2004), other research has shown that selective Ly6C^{high} monocyte depletion does not affect the numbers of Ly6C^{low} monocytes (Geissmann *et al.*, 2010).

A similar division into two monocyte subsets has also been described in rats and pigs (Auffray *et al.*, 2009). The existence of a third murine monocyte subset has recently been proposed, which is Ly6C^{intermediate} but which (unlike most Ly6C^{high} monocytes) is also CD43^{high} (Sunderkötter *et al.*, 2004; Ziegler-Heitbrock *et al.*, 2010).

c) Human monocyte subsets

Monocytes make up about 10% of circulating leucocytes in humans, compared to only 2-4% in mice (Geissmann *et al.*, 2008; Auffray *et al.*, 2009). Human monocytes were initially divided into two subsets, according to whether or not they expressed CD16. More recently, three separate subsets have been described, which can be clearly delineated by their relative intensity of CD14

and CD16 expression, in addition to other cell surface markers (Table 1G) (Ziegler-Heitbrock *et al.*, 2010).

There has been some disagreement in the literature regarding homology between human and murine subsets (Geissmann *et al.*, 2003; Ingersoll *et al.*, 2010). $CD16^{+}$ monocytes were initially labelled as 'pro-inflammatory' and functionally akin to murine 'inflammatory' ($Ly6C^{high}$) monocytes; they demonstrated rapid migration to inflamed tissues and greater release of TNFα and IL-1β (but less IL-10) in response to LPS (Ziegler-Heitbrock, 2007).

Table 1G: Key differences in cell surface marker expression between the three known human monocyte subsets

PHENOTYPE	CLASSICAL (80-90%)	INTERMEDIATE (5-12%)	NON-CLASSICAL (4-7%)
CD14	++	++	+
CD16	-	+	++
HLA-DR	Low	Very high	High
CCR2	+	Intermediate	-
L-selectin	+	-	-
CX3CR1	Low	Intermediate	High
LFA-1 integrin	Low	High	High
CD64	High	Intermediate	Low

Abbreviation: HLA-DR, human leucocyte antigen-DR.
Data derived from (Auffray *et al.*, 2009; Geissmann *et al.*, 2008; Gordon and Taylor, 2005; Grage-Griebenow *et al.*, 2001; Robbins and Swirski, 2010; Tacke and Randolph, 2006; Weber *et al.*, 2000; Wong *et al.*, 2011; Ziegler-Heitbrock *et al.*, 2010).

$CD14^{++}CD16^{-}$ 'classical' monocytes compose the predominant human subset (Soehnlein *et al.*, 2009). They are larger and denser than $CD16^{+}$ monocytes and more closely resemble $Ly6C^{high}$ (and $CD43^{low}$) murine monocytes in phenotype

(Table 1G) (Gordon and Taylor, 2005; Ziegler-Heitbrock *et al.*, 2010). Classical monocytes demonstrate marked chemotaxis in response to MCP-1, with release of IL-10 and IL-6 (Wong *et al.*, 2011).

$CD16^{+}$ monocytes are smaller than classical monocytes and are now defined as two separate subsets. $CD14^{+}CD16^{++}$ 'non-classical' monocytes, as already mentioned, demonstrate strong pro-inflammatory mediator release, but less marked chemotaxis to MCP-1 (they do not express CCR2) (Ziegler-Heitbrock *et al.*, 2010; Wong *et al.*, 2011). They are thought to play a key role in the resolution of inflammation and correlate in phenotype to murine $Ly6C^{low}CD43^{high}$ monocytes (Ziegler-Heitbrock *et al.*, 2010).

The third, $CD14^{++}CD16^{+}$, 'intermediate' monocyte subset is thought to represent an intermediate step in monocyte maturation, between a less mature classical phenotype and the more mature, macrophage-like ($CD16^{++}$) non-classical phenotype (Gordon and Taylor, 2005; Robbins and Swirski, 2010; Wong *et al.*, 2011). Intermediate monocytes demonstrate stronger expression of CD64 and CCR5 and marked release of IL-10, compared to non-classical monocytes (Grage-Griebenow *et al.*, 2001; Tacke and Randolph, 2006; Weber *et al.*, 2000). Intermediate monocytes probably correlate with $Ly6C^{high}CD43^{high}$ murine monocytes (Ziegler-Heitbrock *et al.*, 2010).

d) Pulmonary monocyte influx in animal studies

There is considerable evidence that AMs are indirectly derived from circulating blood monocytes. Studies examining the AMs of mice transplanted with GFP-

labelled bone marrow following whole-body irradiation have demonstrated slow replacement by cells of donor origin (at around one year) (Gordon and Taylor, 2005; Maus *et al.*, 2006). Landsman *et al* carried out a series of adoptive transfer experiments using CD11c/diphtheria receptor toxin (CD11c.DTR) transgenic mice given intra-tracheal diphtheria toxin (DT) to deplete lung macrophages and DCs. They demonstrated that lung (interstitial) macrophages are derived from circulating Ly6C^{low} monocytes (akin to non-classical human monocytes) and that these then evolve to become alveolar macrophages (Landsman *et al.*, 2007; Landsman and Jung, 2007). Furthermore, they presented evidence that Ly6C^{high} monocytes can transform into the Ly6C^{low} subtype and then evolve into macrophages (Landsman *et al.*, 2007).

It has been known for many years that monocyte influx accompanies pulmonary neutrophil accumulation in animal models of lung inflammation, for example, after intra-tracheal LPS administration (Doherty *et al.*, 1994; Henderson, 2003; Maus *et al.*, 2001). AM turnover is higher in the context of inflammation, due to the increased recruitment and apoptosis of monocytes (Janssen *et al.*, 2011; Maus *et al.*, 2006).

Monocytes entering the alveolar space are distinct from AMs and also demonstrate greater CD14 expression and more TNFα release than circulating blood monocytes (Maus *et al.*, 2001; Srivastava *et al.*, 2005). Experiments using labelled neutrophils and monocytes in rats have demonstrated a significant increase in neutrophil and monocyte numbers in both the pulmonary interstitium and, to a greater extent, in BAL (Li *et al.*, 1998). Another group

utilised IV perfluorocarbon nanoemulsions in mice given intra-tracheal LPS to detect both monocyte (and neutrophil) pulmonary influx at 24 hours using ^{19}F magnetic resonance imaging (MRI) (Ebner *et al.*, 2010). Perfluorocarbons are biochemically inert and phagocytosed by monocytes and, to a lesser extent, by neutrophils.

Monocyte and neutrophil influx occur by different mechanisms; whereas monocyte migration is dependent upon E-selectin, β1-integrins (including CD18 and Very Late Antigen-4), VCAM-1 and MCP-1, neutrophil migration occurs via L- and P-selectin, β2-integrins, ICAMs and IL-8 (Henderson, 2003; Li *et al.*, 1998; Maus *et al.*, 2003; Soehnlein *et al.*, 2009).

Neutrophil transmigration across the pulmonary endothelium occurs rapidly (within two hours) and was initially thought to precede later pulmonary monocyte influx (Doherty *et al.*, 1994; Kaplanski *et al.*, 2003; Reutershan, 2005; Soehnlein *et al.*, 2009). In addition, neutropenia was observed to reduce monocyte migration (Doherty *et al.*, 1994; Janardhan *et al.*, 2006). It is now accepted that there is a more complex and interdependent relationship between the transmigration of neutrophils and monocytes. Early monocyte influx, independent of neutrophil migration, was first noted in an experimental model of peritonitis (Henderson, 2003). A later study, using histological analysis of the pulmonary interstitium in rats after intra-tracheal LPS, showed early monocyte influx between 1 and 12 hours and a later, second wave of monocyte influx that peaked after 24 hours (Janardhan *et al.*, 2006).

A key study using *in vivo* confocal imaging of dermis and mesenteric blood vessels demonstrated that, in normal conditions, GFP^{high} (i.e. $Ly6C^{low}$) monocytes 'crawl' along the vascular endothelium with extravasation occurring only rarely (Auffray *et al.*, 2007). This 'patrolling' behaviour is dependent upon the interaction between LFA-1 and CX3CR1, enabling endothelial adherence. An inflammatory stimulus (experimental peritonitis) rapidly causes $Ly6C^{low}$ monocytes to roll rather than crawl, followed rapidly by their extravasation, which is the trigger for subsequent neutrophil chemotaxis and transmigration (Auffray *et al.*, 2007). Studies of lung injury using bone marrow transplantation followed by adoptive transfer have demonstrated that neutrophil influx is potentiated by later CCR2-mediated transmigration of $Ly6C^{high}$ monocytes, which peaks between 24 and 48 hours (Maus *et al.*, 2002; Maus *et al.*, 2003). A second, much later peak (around 7 days) in $Ly6C^{low}$ monocytes is thought to demonstrate their role in the resolution of lung injury, at least in a mouse model of myocardial ischaemia (Nahrendorf *et al.*, 2007).

e) Pulmonary monocyte influx in human studies

Studies in patients who have previously undergone allogeneic bone marrow transplantation have demonstrated gradual replacement of AMs by cells derived from circulating monocytes (Gordon and Taylor, 2005; Nakata *et al.*, 1999; Thomas *et al.*, 1976). Small numbers of alveolar monocyte-like cells have been reported in humans in the absence of inflammation (Frankenberger *et al.*, 2004; Frankenberger *et al.*, 2011).

During inflammation much larger numbers of monocytes transmigrate into the alveoli, with a later peak in the number of AMs (Steinberg *et al.*, 1994). Marked monocyte transmigration has been reported in patients with chronic obstructive pulmonary disease (COPD) (Frankenberger *et al.*, 2004), interstitial lung disease (ILD) (Hance *et al.*, 1985; Hoogsteden *et al.*, 1989; Kiemle-Kallee *et al.*, 1991; Krombach *et al.*, 1996), HIV-related lung disease (Wasserman *et al.*, 1994), cystic fibrosis (CF) (Wright *et al.*, 2009) and ALI (Rosseau *et al.*, 2000). Little is known about the phenotype of monocyte-like cells once they cross the blood-air barrier and before they evolve into AMs; the exact nomenclature used has therefore varied widely. Monocyte-like cells in sputum or BAL are consistently smaller, less granular and less auto-fluorescent compared to mature AMs (Frankenberger *et al.*, 2004; Hance *et al.*, 1985; Hoogsteden *et al.*, 1989; Rosseau *et al.*, 2000; Wasserman *et al.*, 1994; Wright *et al.*, 2009). Compared to mature AMs, monocyte-like cells in the alveolar space demonstrate greater expression of CD14 and CD11b and lower expression of HLA-DR, macrophage markers including CD71 (transferrin receptor), 25F9 and CD206 and the macrophage scavenger receptor MARCO; they also release higher concentrations of pro-inflammatory mediators but have less marked phagocytic activity (Frankenberger *et al.*, 2004; Krombach *et al.*, 1996; Rosseau *et al.*, 2000; Wasserman *et al.*, 1994; Wright *et al.*, 2009).

Similarly, after LPS challenge in healthy subjects, one study reported greater numbers of 'monocytes' in BAL fluid, which were smaller than macrophages and with stronger CD14 expression (O'Grady *et al.*, 2001). Another study reported

greater numbers of 'small macrophages' in sputum (differentiated from AMs simply by size and granularity) (Frankenberger *et al.*, 2011).

Like the equivalent murine subset, human non-classical monocytes also appear to exhibit LFA-1-mediated 'patrolling' of vascular endothelium (Cros *et al.*, 2010; Steppich *et al.*, 2000).

1.6 EXPERIMENTAL MONOCYTE DEPLETION

a) Monocyte depletion in mice

Various means of monocyte depletion are available in mice, for example, using CCR2 knockout mice, giving DT to CD11b-DTR transgenic mice, or using anti-CCR2 monoclonal antibody or intra-peritoneal liposomal clodronate.

Firstly, a group testing $CCR2^{+}$-dependent monocyte depletion reported an unexpected reduction in alveolar neutrophil as well as monocyte accumulation after giving intra-tracheal MCP-1 and LPS to induce experimental lung injury (Maus *et al.*, 2002). Intravital two-photon imaging was used by another group to demonstrate that circulating monocyte depletion by liposomal clodronate reduced transendothelial neutrophil transmigration after intra-tracheal bacterial challenge (Kreisel *et al.*, 2010).

Another study demonstrated significant pulmonary margination of $Ly6C^{high}$ monocytes (by histological analysis) after intra-peritoneal LPS plus high-stretch ventilation (Wilson *et al.*, 2009). Monocyte depletion by liposomal clodronate

significantly reduced both the number of lung-marginated monocytes and the severity of lung injury.

Dhaliwal *et al* (2012) used three methods of monocyte depletion to demonstrate that the beneficial effect on pulmonary neutrophil accumulation persisted at 48 hours and involved reduced neutrophil transendothelial migration into the pulmonary interstitium. Intra-peritoneal liposomal clodronate reduced monocyte and neutrophil influx to the lungs, with reduced lung injury scores, reduced BAL total protein, lung oedema and vascular leak. DT administration in CD11b-DTR mice dramatically reduced pulmonary monocyte and neutrophil migration, the levels of total protein and SDF-1 in BAL fluid and pulmonary leak. Interestingly, the rise in circulating blood neutrophils that also occurred after intra-tracheal LPS administration was also significantly reduced by monocyte depletion. An anti-CCR2 monoclonal antibody also significantly reduced BAL neutrophilia and pulmonary vascular permeability. Furthermore, adoptive transfer of monocytes derived from bone marrow partially rescued LPS-induced pulmonary neutrophil influx (Dhaliwal *et al.*, 2012).

Several studies testing monocyte depletion in mice have therefore shown a significant reduction in experimental lung inflammation, indicating that monocyte depletion may represent a therapeutic option in patients with ALI.

b) Human monocyte depletion by leukapheresis

The term 'apheresis' means collecting a particular constituent of circulating blood. 'Leukapheresis' provides a safe and readily available means of depleting circulating blood leucocytes (including monocytes) in humans. Leukapheresis can be performed using filtration columns, for example granulocyte and monocyte adsorptive apheresis or, more commonly, by centrifugation (Freireich *et al.*, 1965; Linenberger, 2005a). Centrifugation leukapheresis uses a continuous-flow system where blood is diverted from the patient, mixed with citrate anticoagulant and pumped into a spinning centrifuge. Within the centrifuge, cells sediment out according to their density and the speed of the centrifuge, with red blood cells (RBCs) towards the bottom, neutrophils above them, then mononuclear cells (MNCs) and platelets (together making up the 'buffy coat'), while plasma lies at the top (Feige and Sorg, 1984). The MNC component can be collected into a bag and the remainder of the blood is returned to the patient. The system is closed ('vein to vein') and uses disposable tubing to avoid air embolus or infection (Buckner *et al.*, 1969; Linenberger, 2005a).

In current clinical practice, centrifugation leukapheresis is used for reducing leucocytosis in patients with leukaemia, or for collection of peripheral blood stem cells (PBSCs) for autologous transplantation or collection of MNCs for *ex-vivo* processing (e.g. photopheresis for cutaneous T-cell lymphoma), or immune modulation (e.g. DC therapy), before later re-infusion (Linenberger, 2005b; Nguyen *et al.*, 2002; Okafor *et al.*, 2010; Perseghin and Incontri, 2010). PBSC

collection requires prior treatment with G-CSF (with/without chemotherapy) to mobilise sufficient $CD34^+$ cells into the circulation (Arslan and Moog, 2007).

Other types of therapeutic apheresis include red cell exchange in patients with sickle cell anaemia, thrombocytapheresis in patients with thrombocytosis, or in healthy donors to obtain platelet donations, and plasmapheresis to remove circulating immunoglobulin in antibody-mediated diseases (for example, thrombotic thrombocytopaenic purpura, antiphospholipid syndrome and inflammatory polyneuropathies) (Linenberger, 2005b).

There is some evidence that MNC leukapheresis has a beneficial effect in other inflammatory conditions, including inflammatory bowel disease and rheumatoid arthritis (RA) (Cuadrado, 2009; Hidaka *et al.*, 1999; Kohgo *et al.*, 2002; National Institute for Clinical Excellence, 2005). Repeated leukapheresis in patients with RA leads to repopulation of the blood by less activated monocytes and reduced release of pro-inflammatory cytokines (TNFα and IL-1β) (Hahn *et al.*, 1993; Kashiwagi *et al.*, 1998).

Large numbers of MNCs can be collected by large-volume leukapheresis, where at least 3 total blood volumes (TBVs) or $\geq$15 litres of blood are processed (Humpe *et al.*, 2001; Reik *et al.*, 1997). Large-volume leukapheresis does tend to require higher flow rates and thus increase the risk of citrate-related side effects (Arslan and Moog, 2007). Different cell separator machines vary to some extent with regards to MNC yield; choice of programme also affects the degree of contamination of the MNC product by other cells such as platelets and RBCs

(A E Morrison *et al.*, 2000; Strasser *et al.*, 2003; Wolf *et al.*, 2005). Of crucial importance, the side effects of leukapheresis are usually mild and the risk of serious side effects in healthy subjects is minimal (McLeod *et al.*, 1999; Reik *et al.*, 1997; Strauss, 1996).

1.7 HYPOTHESES AND THESIS OVERVIEW

The aim of this work was to therefore build upon the evidence that monocyte depletion in mice ameliorates experimental lung inflammation and, for the first time, translate this into the human setting. As such, my hypotheses were:

A) That inhaled LPS in healthy subjects would consistently and safely produce mild pulmonary and systemic inflammation when compared to saline placebo.

B) That MNC leukapheresis in healthy subjects would consistently and safely remove large numbers of monocytes from circulating blood, with minimal depletion of circulating neutrophils and without itself causing significant inflammatory effects detectable in blood or BAL fluid.

C) That depletion of circulating monocytes by MNC leukapheresis in healthy subjects would ameliorate experimental, LPS-induced systemic and pulmonary inflammation when compared, in a randomised double blind study, to a sham procedure.

My first additional aim was to closely scrutinise monocyte transmigration into the alveolar space after inhaled LPS and to examine the phenotype and function

of monocyte-like cells in BAL fluid. Secondly, I planned to analyse the effects of both inhaled LPS and of MNC leukapheresis on circulating monocyte subsets.

I carried out two preliminary studies (A and B) to address the first two hypotheses respectively. I then performed an RCT in 30 healthy subjects to determine whether monocyte depletion could attenuate experimental lung inflammation in the human setting. The exact aims of each study, as they relate to my hypotheses, are outlined at the beginning of each of the relevant chapters.

Chapter 2 consists of the Methods and Materials used for all three studies. Chapter 3 contains the results of Study A (the first preliminary study). Chapter 4 describes the results of Study B (the second preliminary study). Chapter 5 discusses the results of Study C (the RCT). The final chapter (6) summarises how this work adds to our understanding of the role of monocytes in ALI and discusses the priorities for further research in this area.

CHAPTER 2: MATERIALS AND METHODS

2.1 OVERVIEW

I undertook three consecutive studies, consisting of two preliminary studies followed by an RCT, with the aim of systematically addressing the hypotheses outlined in Chapter 1.

This chapter begins by outlining the overall design of each of the three studies. It goes on to describe the process of subject recruitment, screening and enrolment, followed by details of the procedures performed during each of the three studies. All of the clinical data and biological samples obtained from each subject are specified, with details of the materials used to process the samples and the scientific and laboratory techniques used to generate data. The methods used for statistical analysis are described in detail at the end of the chapter.

2.2 STUDY DESIGN

a) Ethical approval, safety and clinical trial registration

Before commencing, the studies received approval from the Lothian Research Ethics Committees (reference number 09/S1101/27) and the NHS Lothian/University of Edinburgh Research and Development Office. The studies were also registered under an International Standard Randomised Controlled Trial Number (ISRCTN 42695423) before recruitment began. The studies were conducted in accordance with Good Clinical Practice and relevant written

informed consent was obtained before conducting screening visits or study days. Subjects were informed that they could withdraw from the study at any stage and for any reason. Subjects who took part in study days were compensated financially for their time.

A Data Monitoring and Safety Committee (DMSC) was established and consisted of three members (including one statistician) who were otherwise independent from the trial. The DMSC met before any of the studies commenced. They then received regular reports at pre-specified points during and after completion of each study, detailing subject recruitment and enrolment, full blood count (FBC) data for each subject and any expected symptoms or adverse events reported. At each stage, the DMSC members were asked to approve the report before the relevant study could continue with further subject recruitment.

An adverse event (AE) was defined as 'any untoward medical occurrence in a clinical trial subject'. A serious adverse event (SAE) was defined as 'any untoward medical occurrence or effect that results in death, is life-threatening, requires prolongation of hospitalisation or results in permanent/significant disability or incapacity'.

b) Design of Study A

Study A was a single-blinded study of 12 healthy subjects, comparing inhalation of LPS (n=6) with inhalation of saline placebo (n=6) (Figure 2a). An initial screening visit was used to identify potential exclusion criteria and confirm eligibility (details are on Page 83). Six study days were held, with the 12

enrolled subjects randomly allocated to these in pairs, in order of recruitment. On each study day, one subject was allocated to inhalation of 0.9% normotonic saline (placebo) and the second subject to inhalation of LPS.

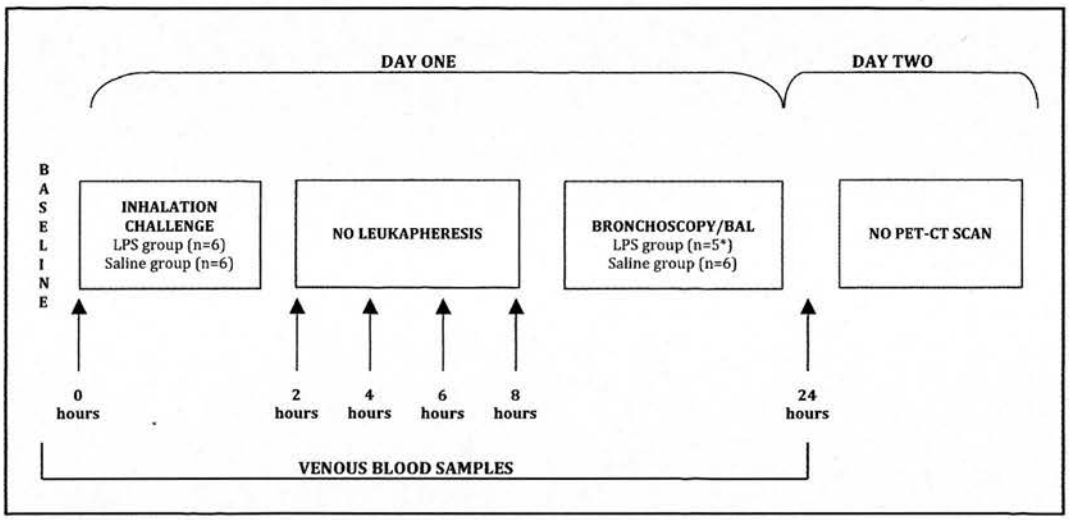


Figure 2a: Design of Study A

Subjects were randomly allocated in a single-blind fashion (according to date of recruitment) to inhalation of either saline placebo or LPS. Leukapheresis was not performed and there was no overnight stay or PET-CT (computerised tomography) scan on the second day.
* One volunteer from the LPS group was unable to tolerate bronchoscopy and therefore BAL was only performed in n=5 subjects from that group.

Routine clinical observations (temperature, HR, BP, SaO_2 and RR) were recorded at baseline and hourly thereafter. Each subject had a blood sample taken at baseline (0 hours) and then inhaled LPS or saline as allocated. Further blood samples were taken at 2, 4, 6 and 8 hours from baseline. Bronchoscopy and BAL were performed between 8 and 9 hours from baseline. Each subject was allowed home later the same evening, returning briefly the following morning (24 hours from baseline) for a final blood sample and medical review.

c) Design of Study B

Study B was an analysis of MNC leukapheresis in a further 6 healthy subjects (Figure 2b).

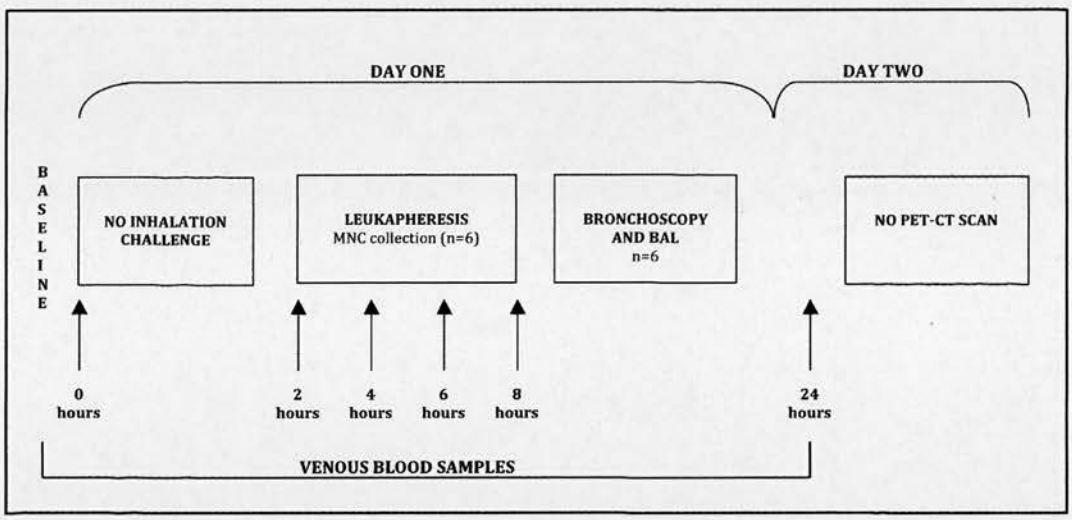


Figure 2b: Design of Study B
The 6 subjects all underwent active MNC leukapheresis. There was no preceding inhalation challenge and there was no overnight stay or PET-CT scan on the second day.

As with Study A, each volunteer had passed an initial screening visit to determine eligibility. Six study days were held, with a single subject attending on each occasion. Routine clinical observations were taken as for Study A, with the exception of BP during leukapheresis (to avoid interrupting blood flow) unless the subject reported feeling light-headed or unwell. Blood samples were taken as for Study A. After the baseline blood sample, subjects were simply observed until the 2-hour time-point when MNC leukapheresis of four total blood volumes (TBVs) commenced. As in Study A, bronchoscopy and BAL were performed between 8 and 9 hours. After a period of observation, the subjects were again allowed home overnight, returning at the 24-hour time-point for a medical review and the final blood sample.

d) Design of Study C

Study C was a randomised double-blinded, placebo-controlled trial of 30 healthy subjects who all received inhaled LPS at baseline, comparing the effects of MNC depletion by leukapheresis (depletion group, n=15) versus a sham procedure (sham group, n=15) (Figure 2c). The subjects had again been screened to determine eligibility prior to their enrolment in the study. Thirty separate study days were held, a single subject attending each study day.

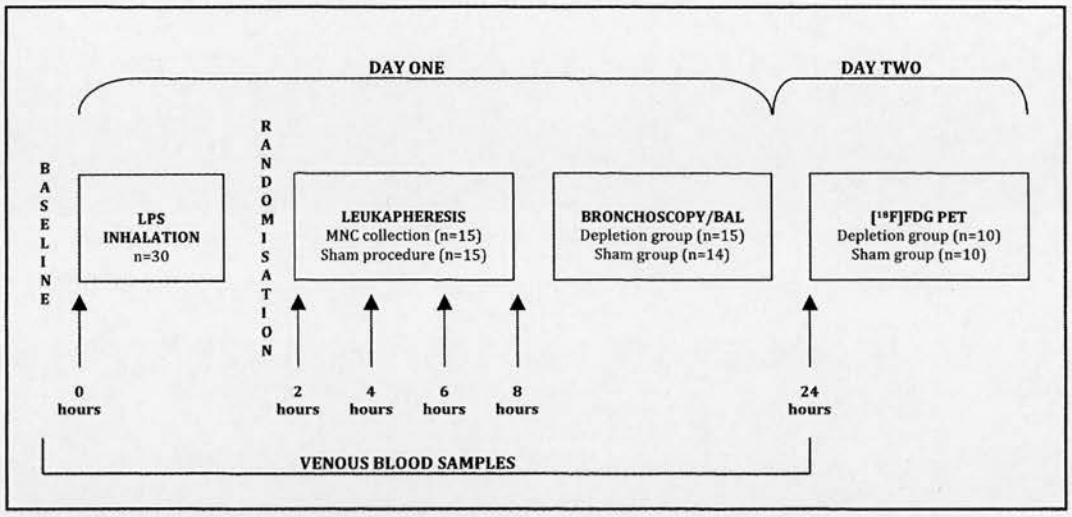


Figure 2c: Design of Study C (the RCT)
All subjects inhaled LPS at baseline. Subjects were then randomised in a double-blind fashion to either active leukapheresis with MNC depletion or a sham procedure. Bronchoscopy and BAL were not performed in one subject from the sham group (due to a >10% fall in FEV_1). As a precaution, all subjects were kept in hospital overnight for observation. A sub-group of 20 subjects underwent a PET-CT scan on the second day.

Routine clinical observations were recorded as for Study B. After the baseline blood sample had been taken, each subject proceeded to LPS inhalation before undergoing randomisation. Leukapheresis (or sham) of four TBVs began 2 hours from baseline. Bronchoscopy and BAL were performed at 9 hours from baseline. As a precaution, the subjects were then kept in hospital overnight for

observation. The following morning, the 24-hour blood sample was taken and the majority of the subjects (n=10 each arm) then underwent PET-CT scanning. After a medical review, the subjects were discharged later that day.

e) Subject recruitment and screening (all studies)

Healthy male volunteers aged between 18 and 40 inclusive were recruited by advertisement within the University of Edinburgh. Responders were sent written information with details of the studies and interested volunteers were then invited to attend a screening visit a maximum of 2 weeks before their intended study day.

The screening visit incorporated a medical history, routine clinical observations, cardio-respiratory examination, spirometry, routine blood tests and a chest radiograph. Exclusion criteria are listed in Table 2A and were categorised as absolute or temporary. If a subject was temporarily excluded from enrolling in a study, they were given the option to return for a second screening visit at a suitable interval (a minimum of two weeks later). No subject was allowed to enrol for more than one study. Childhood asthma did not result in exclusion if it had completely resolved before the subject left primary school. Non-complex congenital valvular heart disease did not result in exclusion if it had been completely corrected during early childhood.

Table 2A: Exclusion criteria at the screening visit

HISTORY	History of chronic illness, including asthma, recurrent upper/lower respiratory tract infection, bronchiectasis, congenital, valvular or ischaemic heart disease, diabetes mellitus, chronic kidney disease or recurrent urinary tract infection
	Presence of any acute medical illness†
	Current medication†
	Smoking within the previous year
	Reported smoking history amounting to >2 pack-years
	Reported alcohol intake >21 units/week†
	Formal blood donation within the previous 12 weeks†
	History of recurrent vasovagal episodes
EXAMINATION	Abnormal pulse rhythm (excluding sinus arrhythmia)
	Persistent tachycardia (HR >90bpm over 15 minutes) †
	Persistent tachypnoea (RR >20/minute over 5 minutes) †
	sBP <90mmHg or dBP <60mmHg†
	Temperature >37.3°C†
	SaO_2 <95% breathing room air
	Presence of any cardiac murmurs
	Presence of added (non-physiological) heart sounds
	Presence of abnormal chest expansion or breath sounds†, wheeze or crackles†
	Insufficient peripheral venous access for leukapheresis
	Significant cardio-respiratory abnormalities on CXR
	FEV_1 or FVC <80% of predicted
	FEV_1/FVC ratio <0.70
INVESTIGATIONS	Abnormal Hb, total WBC†, neutrophil†, lymphocyte†, monocyte† or platelet count; abnormal sodium, potassium or bilirubin† levels
	Raised eosinophils, ALT, random glucose or CRP†
	eGFR <90ml/minute

† denotes a potentially temporary exclusion criterion.
Abbreviations: FVC, forced vital capacity; Hb, haemoglobin; ALT, alanine aminotransferase; eGFR, estimated glomerular filtration rate.

f) Study days (all studies)

Each subject was admitted to the Wellcome Trust Clinical Research Facility (WTCRF) at the Royal Infirmary of Edinburgh (RIE). Routine clinical observations, a brief history and cardio-respiratory examination were repeated to ensure the subject remained well and that the study day could proceed. At each time-point (0, 2, 4, 6, 8 and 24 hours), 10ml venous blood was collected and divided into a 2ml aliquot mixed with heparin sodium and a 7ml aliquot mixed with ethylene diamine tetra-acetic acid (EDTA), which were both taken to the Queen's Medical Research Institute (QMRI) laboratory on ice, plus a 1ml aliquot collected into EDTA and sent to the RIE haematology laboratory to obtain an automated full blood count (FBC).

In studies A and B, assuming the subject remained well 2 hours after completion of bronchoscopy, they were allowed home to return the following morning (at the 24-hour time-point) for a medical review and final blood sample. In Study C, subjects were kept in the WTCRF overnight for observation and were allowed home the following day (after the PET-CT scan, where applicable).

With all three studies, any reported symptoms or possible adverse events were documented. Each subject was contacted within 24 hours after discharge to ensure they remained well and to record any further symptoms. Any symptoms or adverse events were followed up to resolution.

g) Inhalation challenge (Studies A and C)

Prior to inhalation challenge, spirometry (FEV_1 and FVC) was measured at baseline (Vitalograph alpha III spirometer, Vitalograph Ltd, Buckingham, UK). For all spirometry measurements, the best reading from three consistent attempts (with variation <5%) was recorded. The subjects were then instructed how to perform the inhalation challenge, and allowed a 'practice run' using 0.9% saline placebo. Spirometry was then re-checked to ensure there was no subsequent fall in FEV_1 (a drop of ≥10% being the pre-specified cut-off for not proceeding with LPS inhalation). After LPS (or saline placebo) challenge, spirometry was repeated at any time if the subject reported breathlessness, cough or wheeze. If the FEV_1 fell by ≥10%, the subject was observed closely to determine the need for bronchodilator treatment, and spirometry was then repeated at regular intervals until the FEV_1 had returned to within 10% of baseline.

An inhalation-synchronised dosimeter-nebuliser (Spira Elektro 2, Hameenlinna, Finland) connected to a pressurised air cylinder was used for the inhalation challenge (Image 2a). The air cylinder pressure was fixed at 2 bar (200kPa). The nebuliser pot was filled with 1.6ml of sterile 0.9% saline for the practice inhalation in all subjects and also for the placebo inhalation in the 6 control subjects in Study A. For the remaining 6 subjects in Study A and all 30 subjects in Study C, 2mg of LPS (from *E. coli* 026:B6, Sigma, Gillingham, UK) was dissolved in 1.6ml sterile 0.9% saline to form a solution of 1.25mg/ml. This LPS solution was freshly prepared, in sterile conditions, and kept on ice prior to

inhalation. The dosimeter-nebuliser had been calibrated to produce an aerosol of 9.6μl over 0.6 seconds. The subject was instructed to take 5 slow consecutive inhalations from functional residual capacity, holding their breath at maximal inspiration for 5 seconds before exhaling. A total dose of approximately 60μg LPS was therefore inhaled by each subject over the 5 breaths.

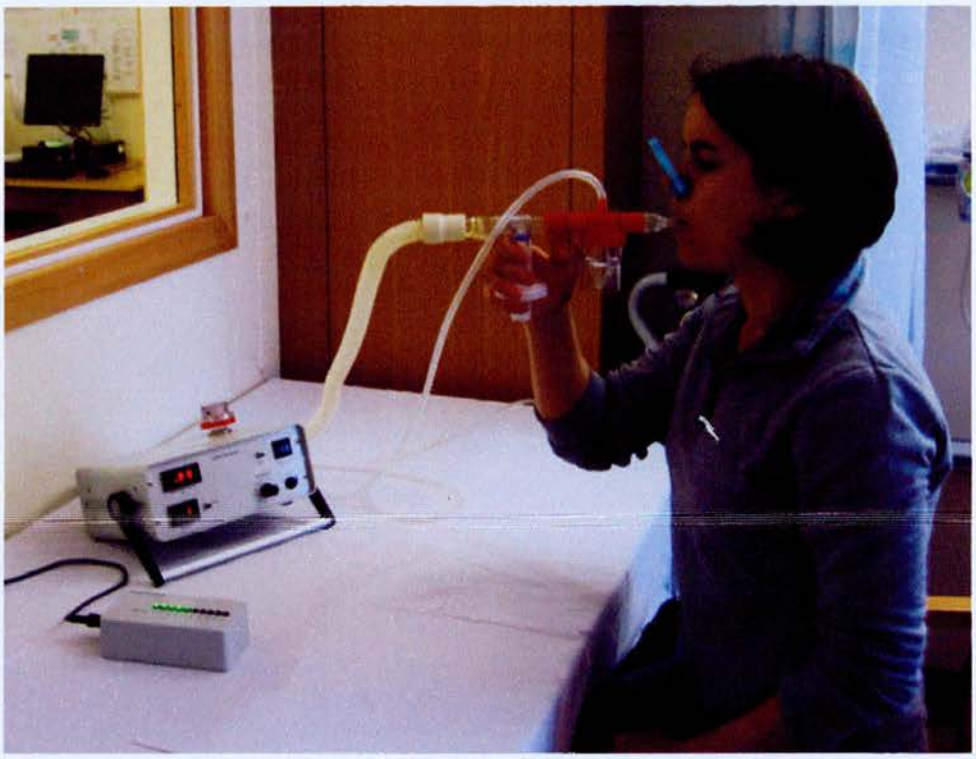


Image 2a: The dosimeter-nebuliser equipment used for inhalation challenge
The dosimeter was programmed to commence nebulisation for 0.6 seconds at an inspired volume threshold of 50ml. There was a flow-meter attached to guide inspiratory flow rate (aiming for 0.5 L/s). The dosimeter display recorded inspired volume for each breath and the total number of inspirations. Disposable nose-clips and exhalation filters were used.

h) Double-blinded randomisation (Study C)

During Study C, the nurse in charge of the RIE Cell Separator Unit (CSU) performed randomisation of each subject once they had completed LPS inhalation challenge. The subject's unique trial number, initials and date of birth

were then entered into a dedicated randomisation website, which generated a randomisation instruction for the CSU nursing staff to follow (MNC depletion or sham leukapheresis). Randomisation was designed to occur in permuted blocks of ten subjects so as to achieve balanced randomisation (equal treatment assignment between study arms) after 10, then 20 and finally all 30 subjects had been recruited.

Members of the research team were blinded as to treatment allocation during Study C and until data entry was complete and validated and the statistician had performed the initial statistical analysis for the primary and main secondary end-points. For the duration of Study C, members of the research team had no access to the RIE laboratory results system upon which the subjects' automated cell count results were anonymously uploaded. During the study, there was a mechanism available within the randomisation website for the research team to 'break the blind' for an individual subject should clinical need arise and knowledge of treatment allocation become imperative. The details of any such un-blinding activity were automatically recorded on the randomisation website.

i) MNC leukapheresis (Studies B and C)

Continuous flow centrifugation leukapheresis was performed in the CSU, using the COBE® Spectra™ Apheresis System MNC Program (version 4.7, CaridianBCT, Lakewood, CO, USA). The COBE® Spectra™ machine automatically calculated an estimated TBV for each subject, based upon their height and weight and according to Nadler's formula (Nadler *et al.*, 1962):

$$\text{Estimated TBV} = 0.3669 * (\text{height in metres})^3 + 0.03219 * (\text{weight in kg}) + 0.6041$$

Acid-citrate-dextrose solution A (ACD-A) was used as an anticoagulant at a ratio of 13.5:1 (whole blood: ACD-A). The required white blood cell interface was established using a 'colorgram' to achieve a collect line haematocrit of 1-2%. In Study B subjects, and in those subjects randomised to the MNC depletion arm of Study C, cell collection then commenced at 1ml/minute. Cell collection was NOT commenced for the 15 subjects in the sham arm of Study C. During Study C, the collection bags and associated tubing were completely covered throughout the procedures (by CSU nursing staff) so that neither the subjects nor members of the research team could determine whether true MNC depletion or a sham procedure was taking place (Image 2b). During Study C, we restricted the total duration of leukapheresis to a maximum of 6.5 hours.

All subjects were encouraged to have a milky drink prior to leukapheresis in order to try to reduce the risk of citrate-induced hypocalcaemia. During Study B, we found that a combination of oral and IV calcium was usually required to minimise or abolish symptoms of hypocalcaemia during leukapheresis. Therefore, in Study C, we gave oral calcium (Calcichew Forte® 1 gram tablets) if needed during the first 2-3 hours, followed by IV administration of 10% calcium gluconate in all subjects, commencing mid-way through the procedure.

Image 2b: Leukapheresis/sham procedure (Study C)
The collection bag and tubing were covered so that it was impossible to tell whether MNCs were being collected (depletion arm) or simply returned to the circulation (sham arm). Randomisation thus remained double-blinded, with neither the subjects nor the investigating team aware of treatment allocation.

At the end of the leukapheresis procedure, in both Study B and in subjects from the depletion arm of Study C, a sample from the MNC collection was placed in EDTA and taken to the haematology laboratory in the RIE for an automated cell count. In Study B, the remainder of the MNC collection was placed on ice for transfer to the laboratory and processed as described below. In Study C, the sample from the MNC collection (for automated cell count) was only retrieved once both the subject and research team had left the CSU, and the remainder of the MNC collection was then discarded, in order to maintain blinding.

j) Bronchoscopy and BAL (all studies)

Bronchoscopy and BAL were performed between 8 and 9 hours from baseline. All subjects were fasted for at least six hours prior to bronchoscopy in order to minimise the risk of aspiration during the procedure. Before proceeding to bronchoscopy, in subjects from Studies A and C (i.e. those who had undergone inhalation challenge), spirometry was re-checked. For safety reasons, bronchoscopy was not performed if the FEV_1 had fallen by >10% from baseline.

All subjects received supplemental oxygen at 2-4 L/minute by nasal cannulae. They were offered sedation in the form of IV midazolam. Topical lidocaine (local anaesthetic) was applied to the throat and, after intubation of the vocal cords, the airways. The RML was identified and the bronchoscope wedged in the medial or lateral segment. 20ml of 0.9% sterile saline was instilled initially, left to dwell for 10 seconds and then aspirated and discarded (the 'bronchiolar' sample). Four separate aliquots of 50ml 0.9% sterile saline (i.e. a total of 200ml) were then instilled, each time allowed to dwell for 10 seconds before aspirating and the BAL fluid retrieved was then collated and stored on ice prior to transfer to the QMRI laboratory.

k) PET-CT scanning (Study C)

10 subjects from Study C (n=5 each group) did not undergo PET-CT scanning due to a delay in scanner installation and calibration. These subjects were simply discharged on the morning of the second day after a medical review and once their final blood sample had been obtained.

The other 20 subjects (randomly assigned, n=10 each group) were allowed water only (otherwise fasted) from midnight until after the scan was completed, to minimise any fluctuations in plasma glucose concentrations. The PET-CT scans were performed in the Clinical Research Imaging Centre (CRIC) at the QMRI, approximately 27 hours from baseline. A Siemens Biograph mCT scanner was used (Siemens Medical Solutions Inc., Knoxville, TN, USA). The CT topogram was used to position the CT and PET beds from the dome of the diaphragm upwards. A baseline thoracic CT scan was carried out for localisation and attenuation correction purposes.

Approximately 200 megabecquerel (MBq) [^{18}F]FDG was drawn up and administered IV; the 60 minute PET scan then commenced immediately, using 'List Mode'. The last 10 minutes of data were reconstructed into a static image for standardised uptake value (SUV) measurement. Nine venous blood samples, each measuring 2ml, were taken at specific time-points during the scan from a freshly placed IV cannula; each sample was collected into a heparinised tube, numbered and stored together in a lead-lined container. Bedside capillary blood glucose measurements were carried out at baseline, mid-point and end of the PET scan to ensure that blood glucose levels remained stable.

Once the scan was complete, the subject was kept in CRIC for a further hour, allowed to eat and drink and then discharged home following medical review. Departmental protocols were followed throughout in order to adhere to ionising radiation regulations.

2.3 LABORATORY MATERIALS AND PROCEDURES

a) Materials and reagents

The vWF enzyme-linked immunosorbent assay (ELISA) kit (catalogue number Ab108918) was from Abcam.

40μm BD Falcon™ Cell Strainers (352340), Anti-mouse and anti-rat Ig BD™ CompBeads (552843 and 552844), BD FACS™ Lysing Solution (349202), BD Falcon™ polystyrene Round-Bottom Tubes (352001), BD Falcon polyprophylene 15ml & 50ml Conical Tubes (352096 and 352070) and BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit, for IL-1β, IL-10, IL-12p70, IL-6, IL-8 and TNFα, (551811) were from Becton Dickinson (BD) Biosciences (Oxford, UK).

The following fluorochrome-conjugated antibodies were from BD Biosciences:

- Anti-human CCR2 Alexa Fluor® (AF)647; BD Pharmingen™, clone 48607, mouse IgG_{2b}, κ (558406).
- Anti-human CD14 allophycocyanin (APC); BD Pharmingen™, clone M5E2, mouse IgG_{2a} κ (555399).
- Anti-human CD16 fluorescein isothiocyanate (FITC); BD Pharmingen™, clone 3G8, mouse IgG_1 κ (556618).
- Anti-human CD3 FITC; BD Pharmingen™, clone UCHT1, mouse IgG_1 κ (555332).
- Anti-human HLA-DR V450; BD Horizon™, clone G46-6, mouse IgG_{2a} κ (561359).

The Quantichrom™ urea assay kit (DIUR-500) was from Bioassay Systems (Hayward, CA, USA).

The following fluorochrome-conjugated antibodies were from Biolegend (Cambridge, UK):

- Anti-human CD11b (integrin alpha M, ITGAM) phycoerythrin (PE)/Cy5; clone ICRF44, mouse IgG_1 κ (301308).
- Anti-human CD14 peridinin-chlorophyll-protein complex (PerCP)/Cy5.5; clone HCD14, mouse IgG_1 κ (325622).
- Anti-human CD16 PE; clone 3G8, mouse IgG_1 κ (302008).
- Anti-human CD163 APC; clone GHI/61, mouse IgG_1 κ (333610).
- Anti-human CD206 (mannose receptor) PE; clone 15-2, mouse IgG_1 κ (321106).
- Anti-human CD25 (IL-2 receptor α) PE; clone BD96, mouse IgG_1 κ (302606).
- Anti-human CD4 APC/Cy7; clone RPA-T4, mouse IgG_1 κ (300518).
- Anti-human CD62L (L-selectin) APC/Cy7; clone DREG-58, mouse IgG_1 κ (304814).
- Anti-human CD64 PE; clone 10.1, mouse IgG_1 κ (305008).
- Anti-human CD71 FITC; clone CY1G4, mouse IgG_{2a} κ (334104).
- Anti-human CX3CR1 FITC; clone 2A9-1, rat IgG_{2b} κ (341606).
- Anti-human Ki-67 PE; clone Ki-67, mouse IgG_1 κ (350504).

NucleoCassettes containing propidium iodide (941-0002) were from ChemoMetec (Allerød, Denmark).

96-well flat-bottom sterile microplates were from Corning (Lowell, MA, USA).

Flow Cytometry Staining Buffer (00-4222-26) and the following fluorochrome-conjugated antibodies were from eBioscience (Hatfield, UK):

- Anti-human 25F9 (mature macrophage marker) AF647; clone eBio25F9, mouse IgG_1 (51-0115-71).
- Anti-human CD127 (IL-7 receptor α) PE/Cy5; clone eBioRDR5, mouse $IgG_1 \kappa$ (15-1278-42).

The HNE and SP-D ELISA kits were from Hycult Biotech (HK319 and HK335).

Dulbecco's Phosphate Buffered Saline (PBS) with Ca/Mg (14040) and Fetal Bovine Serum (FBS) were from Invitrogen (Paisley, UK).

The Reastain Quick-Diff Kit (102164) was from Reagena (Toivala, Finland).

The following ELISA kits were from R&D Systems (Minneapolis, MN, USA):

- Duoset® kits for IL-6 (DY206), IL-8 (DY208), MCP-1 (DY279), RAGE (DY1145), SDF-1α (DY350) and TNFα (DY210).
- Quantikine® kits for CC-16 (DUGB00), MCP-1 (DCP11), MIP-1α (SMA00), MPO (DMYE00), SDF-1α (DSA00) and CD62E (sE-selectin) (DSLE00).
- CD62L (sL-selectin) Immunoassay (BBE4B).

Albumin from Bovine Serum (BSA) (A2153), EDTA (E6758), E-Toxate™ kit (ET0300), Goat serum (G9023), Heparin sodium (H3149), LPS from *E. coli* 026:B6, γ-irradiated, lyophilised powder purified by gel-filtration chromatography (L2654, lot 079K4011) and Tween® 20 (P1379) were from Sigma-Aldrich (Gillingham, UK).

The Pierce bicinchoninic acid (BCA) protein assay kit (23225) was from Thermo Scientific (Pierce Biotechnology, Rockford IL, USA).

Round Base Tubes 3.5ml and caps (212-1821 and 217-7004), for performing radioactivity counts in blood/plasma, were from VWR International Ltd (Lutterworth, UK).

b) Automated cell counts (all studies)

FBC results (incorporating Hb, total and differential WBC count and platelet count) were processed and recorded under anonymised subject number for each time-point. A set of FBC safety parameters operated during each study day. Both LPS inhalation and leukapheresis were expected to alter the FBC results, but the Trial Manager checked each result as it became available and alerted the research team in the event of any Hb level <100 or >180 g/L, total WBC count <2.5 $x10^9$/L, neutrophil count <1 $x10^9$/L or platelet count <50 $x10^9$/L. Any results outwith these FBC safety parameters were also automatically classified as an AE. To maintain blinding, during Study C, the research team otherwise had no access to the FBC results until after preliminary statistical analysis, when the study was un-blinded.

Samples from the MNC collections sent to the RIE haematology laboratory for automated cell counts (Studies B and C) were also processed and recorded under anonymous subject number. During Study C, the research team had no access to these results until after preliminary statistical analysis and study un-blinding.

c) Extracting plasma from peripheral blood samples (all studies)

At each time-point, the 2ml heparinised blood sample and 2ml of the EDTA-treated blood sample were centrifuged for 20 minutes (300g, 20°C). The plasma layer was carefully aspirated by pipette and stored in small aliquots at -80°C. Plasma assays were performed at a later date (see Pages 103 - 104).

d) Staining peripheral blood leucocytes for flow cytometry (all studies)

50µl of Flow Cytometry Staining Buffer was added to each 50µl aliquot of whole blood (from the EDTA sample) and then incubated for 30 minutes (4°C, in darkness) with the appropriate antibody mixture or control (Table 2B). 750µl of FACS Lysing Solution was added to each sample for 20 minutes (at 20°C) to lyse red blood cells. 2ml of PBS was added and the samples centrifuged for 5 minutes (350g, 4°C) before discarding the supernatant. Cell pellets were re-suspended in 500µl FACS Lysing Solution and stored at 4°C prior to flow cytometry.

Table 2B: Fluorochrome-conjugated antibodies for flow cytometry/sorting

STUDY	CELLS OF INTEREST	SAMPLES ANALYSED	ANTIBODIES
Study A	Monocytes Neutrophils	Blood	CD14 PerCP/Cy5.5, CD16 PE-TR, HLA-DR V450, CCR2 AF647, CX3CR1 FITC, CD11b PE/Cy5, CD62L APC/Cy7, CD64 PE
	T lymphocytes	Blood BAL	CD3 FITC, CD4 APC/Cy7, CD25 PE, CD127 PE/Cy5
	Macrophages Neutrophils Monocyte-like cells	BAL	HLA-DR V450, CD14 PerCP/Cy5.5, CD16 PE-TR, CD206 PE, CD163 APC
Study B	Monocytes Neutrophils Monocyte-like cells	Blood MNC collection BAL	HLA-DR V450, CD14 PerCP/Cy5.5, CD16 PE-TR
	T lymphocytes	Blood BAL	CD3 FITC, CD4 APC/Cy7, CD25 PE, CD127 PE/Cy5
Study C	Monocytes Neutrophils	Blood	CD14 PerCP/Cy5.5, CD16 PE, HLA-DR V450
	Macrophages Neutrophils Monocyte-like cells	BAL	CD14 PerCP/Cy5.5, CD16 PE, HLA-DR V450, CD163 APC, CD71 FITC
	Macrophages Neutrophils Monocyte-like cells	BAL	CD14 PerCP/Cy5.5, CD16 FITC, HLA-DR V450, CD206 PE
	Monocyte-like cells	BAL	CD14 PerCP/Cy5.5, CD16 FITC, Ki-67 PE
	T lymphocytes	Blood BAL	CD3 FITC, CD4 APC/Cy7, CD25 PE, CD127 PE/Cy5
	Monocyte-like cells (Flow sorting)	BAL	CD14 APC, CD16 PE, HLA-DR V450

e) Processing the MNC collection (Study B)

The cell collection was divided into 50ml aliquots and centrifuged for 20 minutes (350g, 4°C, no acceleration/brake). The top layer, consisting mainly of plasma and platelets, was then aspirated and discarded. Red blood cells in the remaining cell solution were lysed using ice-cold hypotonic and hypertonic saline (as described above). The cell solution was centrifuged for 6 minutes (350g, 4°C) and the supernatant discarded. The cell pellet was washed in 50ml

PBS and then re-suspended in 10ml 0.1% BSA in PBS with Ca/Mg. Automated total and viable cell counts were obtained using a NucleoCounter (ChemoMetec (Allerød, Denmark).

f) Staining cells from the MNC collection for flow cytometry (Study B)

100µl aliquots of MNC cells, in 100µl 0.1% BSA in PBS with Mg/Ca, were incubated with the relevant antibodies (Table 2B) or control for 30 minutes (4°C, in darkness). Lysis and wash steps were performed as for peripheral blood leucocytes and the cell pellets were re-suspended in FACS Lysing Solution and stored at 4°C for flow cytometry.

g) BAL cell counts and extraction of BAL fluid supernatant (all studies)

The volume of BAL fluid was measured and recorded for each subject. Automated total and viable cell counts were obtained using the NucleoCounter. BAL fluid was filtered through 40µm cell strainers into 50ml Falcon tubes and then centrifuged for 10 minutes (300g, 4°C, no brake). The BAL supernatant was carefully aspirated using a pipette and stored in aliquots at -80°C. BAL supernatant assays were performed at a later date (see below).

The cell pellet was re-suspended in 50ml PBS, centrifuged for 10 minutes (300g, 4°C) and the supernatant discarded. The BAL cells were then re-suspended in 0.1% BSA in PBS with Ca/Mg, at a concentration of $1x10^6$ cells/ml. Four cytospins were prepared from each sample, using aliquots of $4 x10^4$ cells suspended in 200µl of fetal calf serum. Once dry, cytospins were fixed and

stained with Reastain Quick-Diff. Differential cell counts were performed by two independent blinded observers (including one pathologist) at x400 magnification, counting 500 nucleated cells for each subject. Cells were defined as AMs, neutrophils or MNCs (lymphocytes and monocyte-like cells).

h) Staining BAL cells for flow cytometry (all studies)

The BAL cells were again centrifuged for 5 minutes (300g, 4°C) and the supernatant discarded. The cell pellet was re-suspended for 45 seconds in 5ml ice-cold 0.2% NaCl before adding 5ml 1.6% ice-cold NaCl for a further 45 seconds. The cells were washed in PBS, centrifuged for 5 minutes as before and the supernatant discarded, before re-suspending in 0.1% BSA in PBS with Ca/Mg at 1 $x10^6$ cells/ml.

100µl aliquots of BAL cells were mixed with an equal volume of 0.1% BSA in PBS with Ca/Mg and incubated for 30 minutes (4°C, in darkness) with the relevant antibody mixtures (Table 2B). 750µl of FACS Lysing Solution was added for 20 minutes (20°C) and the samples were then washed in 2ml PBS, centrifuged for 5 minutes (300g, 4°C) and the supernatant discarded. Cells were re-suspended in 500µl of FACS Lysing Solution and stored at 4°C before flow cytometry.

i) Staining BAL cells for flow sorting (Study C)

The remaining BAL cells were incubated with the relevant antibodies (Table 2B) for 30 minutes (4°C, in darkness). The sample volume was adjusted to 50ml

using PBS and the cells were centrifuged as before and the supernatant discarded. The cell pellet was resuspended in 1% autologous serum in PBS and flow sorting was then performed immediately.

j) Flow cytometry (all studies)

A BD SORP LSRFortessaII cell analyser (calibrated daily) and FACSDiva software (BD Biosciences) were used for flow cytometry. A minimum of 150,000 events was captured for each sample. Compensation was calculated automatically but verified manually for each antibody, using anti-mouse or anti-rat Ig CompBeads. Voltage parameters were kept consistent throughout each study.

Multiple experimental controls were employed. Unstained samples were prepared for each sample/time-point. Single antibody stains and isotype controls were also used for blood samples (at baseline) and for BAL and MNC collection samples. Fluorescence-minus-one controls were used for CCR2 and CX3CR1 staining (these excluded the antibody of interest, replacing it with the appropriate isotype control).

k) Flow sorting (Study C sub-group)

Flow sorting was performed using a BD FACSAria II with FACSDiva software to collect separate populations of AMs and monocyte-like cells. Each cell population was collected into 10% autologous serum in PBS and checked for purity. Cells were incubated with or without 100nM dexamethasone in standard

tissue culture conditions for 24 hours. Cell culture supernatants were extracted and frozen in aliquots at -80°C.

l) Analysing data from flow cytometry (all studies)

Data generated by flow cytometry was analysed using FlowJo version 9.1 (TreeStar, Ashland, OR, USA). For whole blood samples, differences in cellular size and granularity visible on forward scatter-area (FSC-A) versus side scatter-area (SSC-A) plots were used to gate on populations of monocytes, lymphocytes and neutrophils. Within the monocyte gate, HLA-DR$^+$ cells were selected (thus excluding any HLA-DR$^-$ neutrophils) and the proportions of classical (CD14^{++}CD16$^-$), intermediate (CD14^{++}CD16$^+$) and non-classical (CD14$^+$CD16^{++}) monocytes were determined (see Figure 3d, Page 120). For each monocyte subtype, the expression of CD11b, L-selectin, CD64, CCR2, CX3CR1 and HLA-DR was defined by calculating relative geometric mean fluorescence (GMF) intensity; relative expression was categorised as low, intermediate or high. Within the neutrophil gate, HLA-DR$^-$ cells were selected and relative expression of CD11b, CD16, L-selectin and CD64 was determined. For lymphocytes, CD3$^+$ cells were selected from within the lymphocyte gate, and the relative proportions of CD4+ and CD8+ T-lymphocytes were calculated. Regulatory T (T_{reg}) cells were defined as CD25$^+$CD127$^-$ cells within the CD3$^+$CD4$^+$ T-cell population (see Figure 3f, Page 123).

For monocytes within the MNC collection, CD14 and CD16 expression in HLA-DR$^+$ monocytes were used to differentiate the classical, intermediate and non-

classical subtypes and calculate the relative proportion of each (see Figure 4d, Page 157).

In BAL fluid, plots of FSC-A versus SSC-A were also used to gate on AM, neutrophil, lymphocyte and monocyte-like cell populations (see Figure 3i, Page 129). Within the monocyte-like cells gate, HLA-DR$^-$ cells (neutrophils) were excluded and the remaining HLA-DR$^+$ cells were further distinguished into two distinct subtypes (CD14^{++}CD16$^-$ and CD14^{++}CD16$^+$) (see Figure 3j, Page 130).

m) Measuring protein and urea concentrations (all studies)

Protein concentrations were assayed in plasma at baseline and at 8 hours and in BAL fluid supernatant (9 hours). The colorimetric BCA protein assay was conducted according to the manufacturer's instructions. This test relies upon the protein-mediated reduction of Cu^{2+} to Cu^{1+} in alkaline conditions. Molecules of BCA chelate with Cu^{1+} to form a water-soluble purple-coloured complex whose absorbance at 562nm displays a linear correlation with protein concentration (between 20-2000 μg/ml). For each plate analysed, a standard curve was generated from known concentrations of BSA and the optical density (OD) readings for standards and samples were recorded using a microplate reader.

Urea concentrations were assayed in 8-hour plasma samples and in BAL fluid supernatants. The Quantichrom™ kit employs a chromogenic reagent that complexes with urea to form a yellow-coloured complex whose absorbance at 520nm is linearly correlated with urea concentration. Plasma samples (5μl

aliquots) were incubated with working reagent for 20 minutes and the OD at 520nm was compared to that from two standard concentrations (0mg/dL and 50mg/dL). For BAL fluid supernatant samples, the 'low urea' method was used; 50µl aliquots of sample were incubated with working reagent for 50 minutes and the OD at 430nm was measured (standard concentrations were 0mg/dL and 5mg/dL).

The following equation was used to determine urea concentrations in the samples (where DF is the dilution factor; high standard concentration was 50 mg/dL for plasma and 5 mg/dL for BAL fluid supernatant):

$$[\text{urea, mg/dL}] = \frac{(\text{OD sample} - \text{OD zero standard})}{(\text{OD high standard} - \text{OD zero standard})} \times DF \times [\text{high standard}]$$

Urea concentrations in plasma and BAL supernatant were then used to calculate the volume of epithelial lining fluid (ELF) (Rennard *et al.*, 1986):

$$\text{ELF volume, ml} = \frac{[\text{BAL urea, mg/ml}] \times \text{BAL volume, ml}}{[\text{plasma urea, mg/ml}]}$$

Dividing the volume of BAL supernatant by the volume of ELF gives a dilution factor for each BAL sample. Cytokine and protein concentrations in BAL supernatant can then be adjusted according to the degree of BAL dilution.

n) Enzyme-linked immunosorbent assays (all studies)

Samples of plasma at baseline and 24 hours were sent to the RIE biochemistry laboratory for automated analysis of CRP levels. Other ELISAs were performed in the QMRI laboratory as per manufacturer's instructions (Table 2C).

Table 2C: Inflammatory cytokines and markers of cellular injury measured in plasma, BAL supernatant and flow-sorted cultured BAL cell supernatants

CYTOKINE OR CELL MARKER	TYPE OF ASSAY	SAMPLES TESTED	LOWER LIMIT OF DETECTION
STUDIES A AND B			
IL-6	ELISA Duoset®	BAL	9.4 pg/ml
IL-8	ELISA Duoset®	BAL	31.3 pg/ml
IL-10	ELISA Duoset®	BAL	31.3 pg/ml
MCP-1	ELISA Duoset®	BAL	15.6 pg/ml
TNF-α	ELISA Duoset®	BAL	15.6 pg/ml
STUDY C			
CC-16	ELISA Quantikine®	BAL & plasma	70.0 pg/ml
IL-1β	CBA kit	BAL* & plasma	7.2 pg/ml
IL-6	CBA kit	BAL* & plasma	2.5 pg/ml
IL-8	CBA kit	BAL* & plasma	3.6 pg/ml
IL-10	CBA kit	BAL* & plasma	3.3 pg/ml
IL-12p70	CBA kit	BAL* & plasma	1.9 pg/ml
MCP-1	ELISA Quantikine®	BAL & plasma	5.0 pg/ml
MIP-1α	ELISA Quantikine®	BAL & plasma	10.0 pg/ml
MPO	ELISA Quantikine®	BAL & plasma	0.1 ng/ml
HNE	ELISA kit	BAL & plasma	0.4 ng/ml
RAGE	ELISA Duoset®	BAL & plasma	62.5 pg/ml
SDF-1α	ELISA Quantikine®	BAL & plasma	18.0 pg/ml
sE-selectin	ELISA Quantikine®	BAL & plasma	9.0 pg/ml
sL-selectin	ELISA kit	BAL & plasma	0.3 ng/ml
SP-D	ELISA kit	BAL & plasma	6.3 ng/ml
TNFα	CBA kit	BAL* & plasma	3.7 pg/ml
vWF	ELISA kit	BAL & plasma	2.5 mU/ml

* includes BAL fluid supernatant and supernatants from flow-sorted cultured BAL cells.

The sE-selectin ELISA was performed on heparinised plasma (not EDTA, as it is a Ca^{2+}-dependent molecule); all other ELISAs were performed on plasma from

EDTA-treated blood. BAL fluid supernatant results were also adjusted for BAL dilution (i.e. the volume of ELF) using the urea method, as detailed above (Rennard *et al.*, 1986).

o) LPS assay (Study C)

LPS levels were measured in BAL fluid supernatant samples from Study C. The semi-quantitative E-Toxate™ assay is based upon the *Limulus* Amoebocyte Lysate (LAL) test and was performed according to the manufacturer's instructions.

p) Measuring [^{18}F]FDG activity in plasma (Study C sub-group)

Whole blood and plasma radioactivity were measured for each blood sample using a Packard Cobra II Auto-Gamma counting system (GMI Inc., Ramsey, MN, USA). The radioactivity of each aliquot of whole blood was counted over 1 minute. The blood samples were then centrifuged (300g, 20°C, 10 minutes) and the plasma extracted into separate, labelled tubes. The radioactivity of each aliquot of plasma was then counted over 1 minute. The radioactivity counts for whole blood and plasma samples were individually corrected for sample weight, background radioactivity (calculated from an empty control tube) and decay time to give decay-corrected whole blood or plasma activity (Bq/ml).

Apollo® quantitative lung imaging software (VIDA Diagnostics, Coralville, IA, USA) was used to segment lungs and lobes within the CT images. All lung segmentation volumes were shrunk by 25% to avoid contamination from

myocardium (which demonstrates very high FDG uptake). For each subject, the [^{18}F]FDG activity in lung tissue and in plasma was used to generate time-activity curves, construct Patlak plots using linear regression and thereby calculate influx constant (K_i) (Patlak *et al.*, 1983; Patlak and Blasberg, 1985). The Patlak plot is a graphical tool for analysing the uptake of a radioactive tracer in a two-compartmental model, with plasma representing the reversible compartment. Upon cellular uptake (for example, within the lungs), [^{18}F]FDG becomes phosphorylated by hexokinase and is irreversibly trapped within the cell (Harris and Schuster, 2007). After sufficient time has elapsed to create equilibrium between the two compartments, the curve of the Patlak plot becomes linear, with the slope equivalent to K_i (Chen *et al.*, 2004). K_i corrected for intercept was used to adjust for volume of distribution (Chen *et al.*, 2006; Jones *et al.*, 1997).

Mean SUVs were calculated from the PET images obtained during the final 10-minute frame. SUV is the semi-quantitative ratio of tissue radioactivity ([^{18}F]FDG uptake) within the region of interest to the injected [^{18}F]FDG activity, adjusted for body weight (Thie, 2004).

2.4 STATISTICAL ANALYSIS

When designing Study C, a power calculation was performed to determine the minimum subject numbers required to achieve a 90% chance of detecting a clinically and statistically significant reduction in blood neutrophil count. Based

on previous work, LPS inhalation was expected to cause an increment of about 5.5 $x10^9$/L in blood neutrophil count (Shyamsundar *et al.*, 2009); a reduction of ≥2 $x10^9$/L was deemed to be significant, based on a predicted standard deviation of 1.4 $x10^9$/L. The minimum subject number was calculated at n=24; a pre-specified total of 30 subjects were enrolled into Study C to ensure that any early withdrawals from the study did not compromise the statistical power.

For all three studies, the majority of data analysis was undertaken using GraphPad Prism version 4.0 (GraphPad Software, La Jolla, CA, USA). A *P* value of <0.05 was considered statistically significant. 95% confidence intervals (CI_{95}) are given where appropriate. Data with a normal distribution were expressed as mean (standard deviation, SD) and analysed by 2-sample t-test, or paired t-test (for paired data). Data that were not normally distributed were expressed as median [interquartile range, IQR] and analysed by Mann Whitney *U* (MWU) test (unpaired data) or Wilcoxon signed rank test (paired data). Pearson's test was used to calculate correlations. Differences in the frequency of adverse events between study groups were analysed using Fisher's exact test.

Ranked tests (i.e. MWU or Wilcoxon signed rank) were used to analyse concentrations of cytokines and other plasma/BAL markers; concentrations that were undetectable (below the lower limit of detection of the assay) were assigned an arbitrary ranked value equivalent to 50% of the lower limit of detection prior to statistical analysis.

In Study C, cytospin counts of BAL cell populations (from two blinded, independent observers) were compared by the study statistician. Observer agreement was displayed using Bland-Altman plots, aiming for a 95% limit of agreement. According to these limits, the initial counts for 1 subject did not demonstrate sufficient observer agreement and a re-count was performed (which then demonstrated satisfactory consistency between the two counts).

The effects of treatment (in Study A and Study C) on changes in parameters by 8 hours (compared to baseline), or by 24 hours, were assessed using analysis of covariance (ANCOVA) with baseline value as covariant (Minitab, version 15, Minitab Inc., Pennsylvania, USA). Repeated-measures ANCOVA was used to assess the effects of treatment when comparing changes in a variety of parameters between baseline and 8 hours, while incorporating the intermediate (2, 4 and 6-hour) time-points (SPSS version 17, IBM Corporation, New York, USA).

CHAPTER 3: RESULTS OF STUDY A

3.1 OVERVIEW

This chapter reports the results of Study A, which compared inhalation of LPS with inhalation of saline placebo in a group of twelve healthy volunteers (n=6 in each group).

The main aims of this study were:

- To ensure the **safety and efficacy of LPS inhalation** as a model of pulmonary and systemic inflammation in healthy subjects
- To determine the presence and phenotype of **monocyte-like cells in BAL fluid**, in particular after LPS inhalation

Data on recruitment and subject demographics at baseline will be presented first, followed by the effects of LPS inhalation on symptoms, adverse events and clinical parameters, compared to saline placebo. I shall then describe in detail the effects of LPS inhalation on FBC parameters (especially blood neutrophil counts), blood monocyte subsets and T_{reg} cells. Finally, the effects of LPS inhalation on the cellular, protein, cytokine and inflammatory marker content of BAL fluid will be presented, with a particular emphasis on the proportions and phenotype of monocyte-like cells in BAL fluid when compared to control subjects.

3.2 SUBJECTS

a) Subject recruitment

Six subjects were recruited into each group. Recruitment, enrolment and follow-up are illustrated in Figure 3a. There were no withdrawals from the study; however, one subject from the LPS group was unable to tolerate bronchoscopy despite midazolam sedation and the procedure was abandoned without intubation of the vocal cords. BAL data were therefore only available for 5 out of the 6 subjects within the LPS group.

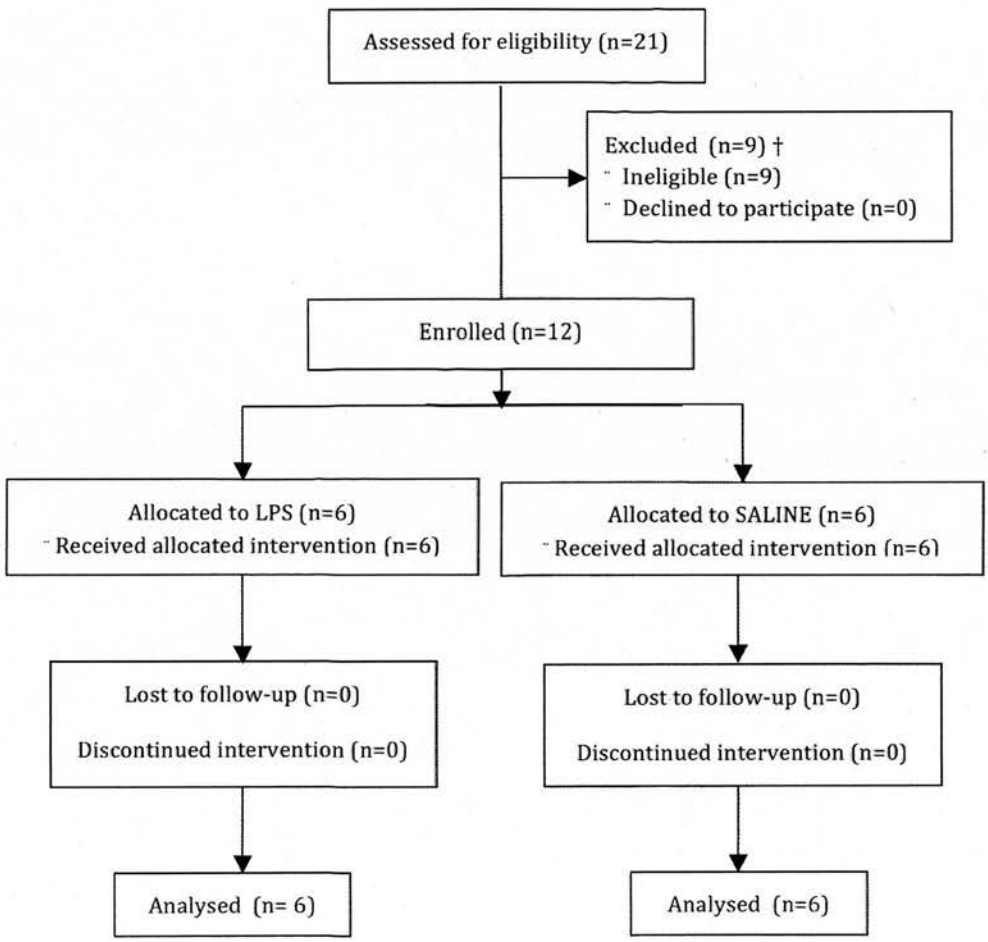


Figure 3a: CONSORT* diagram illustrating volunteer recruitment, enrolment and follow-up during Study A

† Reasons for exclusion: abnormal total/differential WBC (n=5), ALT (n=1) or bilirubin (n=1); current medication (n=1); recent formal blood donation (n=1).

* (CONSORT guidelines are described in Schulz *et al.*, 2010; Schulz *et al.*, 2010)

b) Subject demographics

The baseline characteristics of subjects were very similar between the two groups (Table 3A). All subjects successfully completed inhalation challenge to receive an approximate dose of 60μg of either LPS or saline placebo.

Table 3A: Subject characteristics at baseline

BASELINE CHARACTERISTICS	SALINE GROUP	LPS GROUP
Age (years)	24.7 (5.0)	24.8 (6.2)
Ethnicity	5/6 Caucasian	5/6 Caucasian
Height (metres)	1.81 (0.14)	1.77 (0.04)
Weight (kg)	81.9 (17.2)	79.6 (20.7)
FEV_1 (litres)	4.69 (1.21)	4.30 (0.41)
FVC (litres)	5.61 (1.36)	5.10 (0.58)
Temperature (°C)	36.3 (0.3)	36.3 (0.5)
Heart rate (beats/minute)	71 (12)	71 (10)
Oxygen saturations (%)	98 (2)	98 (1)

Data are presented as mean (SD); n=6 in each group. Subject characteristics were very similar in the two groups.

c) Symptoms and adverse events

There were no reported SAEs and there were no FBC results that fell outside of the pre-specified safety parameters (see Page 96). All reported symptoms were attributable to either LPS inhalation or to bronchoscopy and all resolved promptly (Table 3B).

One subject from the saline group reported transient pyrexial symptoms occurring about 15 hours from baseline; these had fully resolved by the following morning and were attributed to a recognised adverse effect of BAL.

Table 3B: Reported symptoms and adverse events during Study A

SYMPTOM/ADVERSE EVENT	SALINE GROUP	LPS GROUP
Sore throat	3 (50)	4 (67)
Cough	1 (17)	2 (33)
Pyrexia (temperature >37.4 °C)	1 (17)	5 (83) †
Myalgia	0	1 (17)

Data are presented as frequency (percentage); n=6 in each group; † only 2 subjects in the LPS group actually reported symptoms of pyrexia; no subjects reported breathlessness, wheeze or chest discomfort.

3.3 THE EFFECTS OF LPS ON CLINICAL PARAMETERS

LPS inhalation was associated with a significant rise from baseline in mean maximum temperature, when compared to saline inhalation (mean temperature 36.3 (SD 0.5)°C at baseline and maximum 37.7 (0.5)°C in the LPS group, versus 36.3 (0.3)°C and 37.0 (0.4)°C respectively in the saline group; P=0.03) (Figure 3b). The mean maximum temperature of subjects in the saline group tended to rise during the course of the day, consistent with natural diurnal patterns in human body temperature (Lowry, 2003; Martinez-Nicolas *et al.*, 2011).

Inhalation of LPS also caused a significant increment in mean maximum HR when compared to saline placebo (mean HR 71 (SD 10) minute^{-1} at baseline and maximum 97 (14) minute^{-1}, versus 72 (12) minute^{-1} and 76 (12) minute^{-1} respectively in the saline group; P=0.003) (Figure 3b).

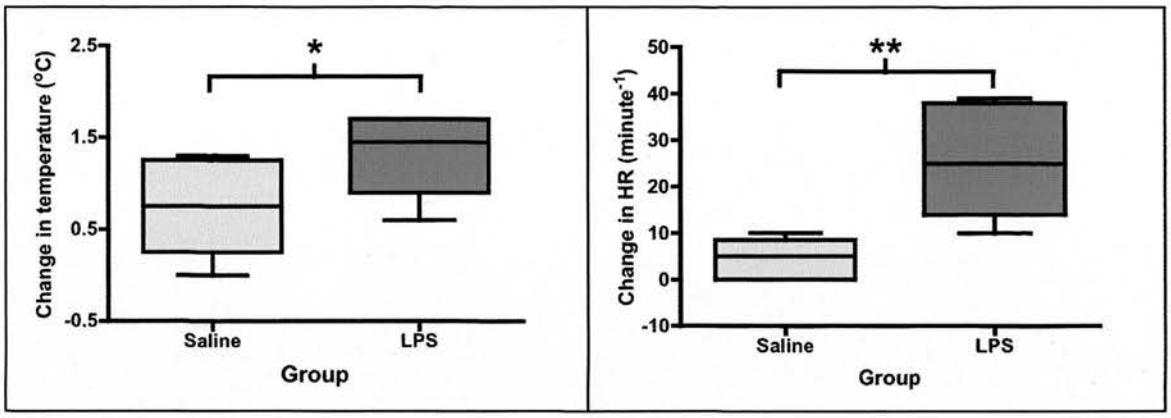


Figure 3b: Maximal changes in temperature and heart rate during the study
Data are displayed as box (median/IQR) and whiskers (range); n=6 each group; between-groups statistical analysis was by ANCOVA, with baseline value as covariate; *P<0.05; **P<0.005.

There were very small changes from baseline in mean maximum RR and mean minimum SaO_2 that were felt unlikely to be of clinical significance and which did not vary between the saline and LPS groups (Table 3C). In both groups, neither LPS nor saline inhalation appeared to have any significant effect on mean FEV_1 and mean FVC values at 8 hours (Table 3C).

Table 3C: Maximal changes in other clinical parameters throughout the study

PARAMETER	GROUP	BASELINE	MAXIMUM/MINIMUM	*P* VALUE
RR (minute^{-1})	Saline	13 (2)	17 (2)	0.83
	LPS	13 (1)	17 (2)	
SaO2 (%)	Saline	98 (2)	97 (2)	0.49
	LPS	98 (1)	96 (3)	
FEV_1 (litres)	Saline	4.69 (1.21)	4.68 (1.14)	0.36
	LPS	4.30 (0.41)	4.22 (0.42)	
FVC (litres)	Saline	5.62 (1.36)	5.52 (1.41)	0.99
	LPS	5.10 (0.58)	5.00 (0.61)	

Data are expressed as mean (SD), n=6 in each group; between-groups statistical analysis was by ANCOVA, using baseline as covariate.

3.4 THE EFFECTS OF LPS INHALATION AS MEASURED IN BLOOD

a) Blood neutrophil counts

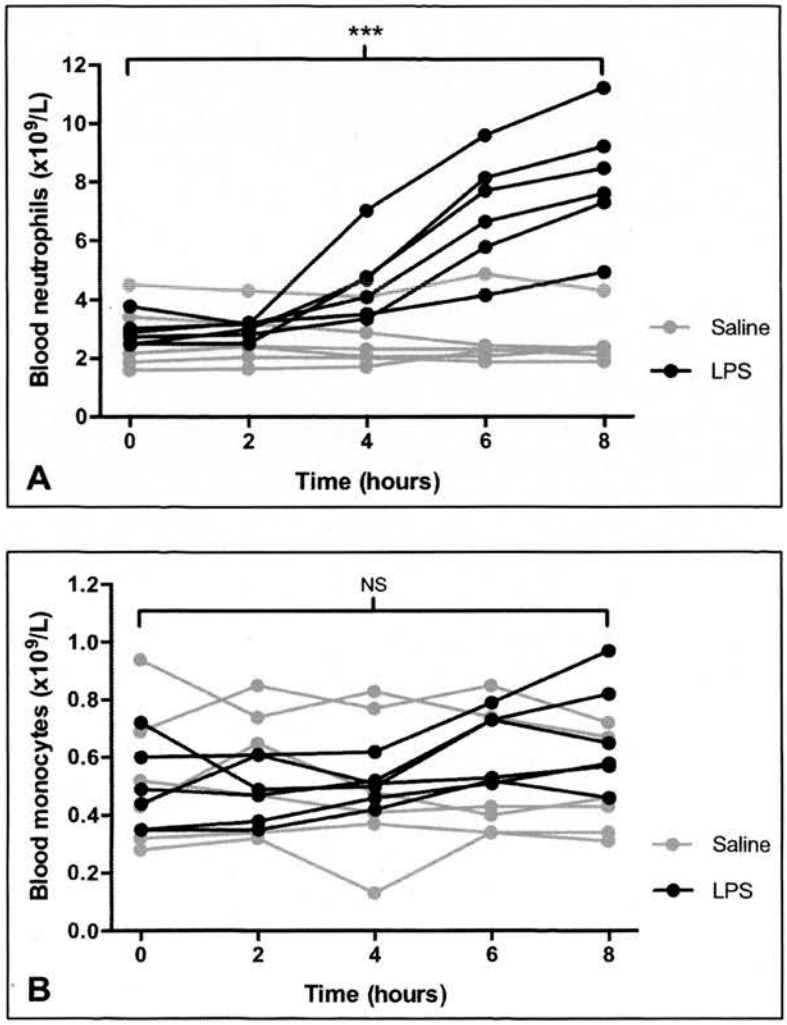
LPS inhalation was associated with a significant rise in mean peripheral blood neutrophil count by 8 hours that was not evident in the saline group subjects (mean blood neutrophil count 2.90 (SD 0.49) $x10^9$/L at baseline and 8.14 (2.11) $x10^9$/L at 8 hours in the LPS group, versus 2.68 (1.09) $x10^9$/L and 2.56 (0.88) $x10^9$/L in the saline group; P=0.0002) (Figure 3c, Panel A). By 24 hours, mean circulating blood neutrophil counts were at similar levels in the two groups (falling to 6.25 (SD 3.00) $x10^9$/L in the LPS group and rising to 5.46 (4.48) $x10^9$/L in the saline group; P=0.78 for between-groups comparison with baseline). There was a particularly marked rise in peripheral blood neutrophil count (to 13.86 $x10^9$/L) in the subject from the saline group who had reported transient pyrexial symptoms post-BAL. He was asymptomatic with normal clinical parameters by the time of medical review at the 24-hour time-point.

b) Blood monocyte counts

There was a trend towards a rise in blood monocyte count at 8 hours in the LPS group that did not quite reach significance (mean blood monocyte count 0.49 (SD 0.15) $x10^9$/L at baseline and 0.68 (0.19) $x10^9$/L at 8 hours in the LPS group, versus 0.53 (0.25) $x10^9$/L and 0.49 (0.17) $x10^9$/L in the saline group; P=0.05 (Figure 3c, Panel B). By 24 hours, mean blood monocyte count was 0.57 (SD 0.17) $x10^9$/L in the LPS group versus 0.67 (0.29) $x10^9$/L in the saline group (P=0.55 for between-groups comparison with baseline).

c) Blood lymphocyte counts

LPS inhalation had no effect on blood lymphocyte counts between baseline and 8 hours when compared to the saline group (mean blood lymphocyte count 2.06 (SD 0.51) $x10^9$/L at baseline and 1.88 (0.62) $x10^9$/L at 8 hours, versus 1.96 (0.55) $x10^9$/L and 2.21 (0.41) $x10^9$/L in the saline group; P=0.17) (Figure 3c, Panel C). Mean blood lymphocyte counts remained similar at 24 hours in both groups (1.97 (SD 0.48) $x10^9$/L in the LPS group versus 1.92 (SD 0.42) $x10^9$/L in the saline group; P=0.96 for between-groups comparison with baseline).



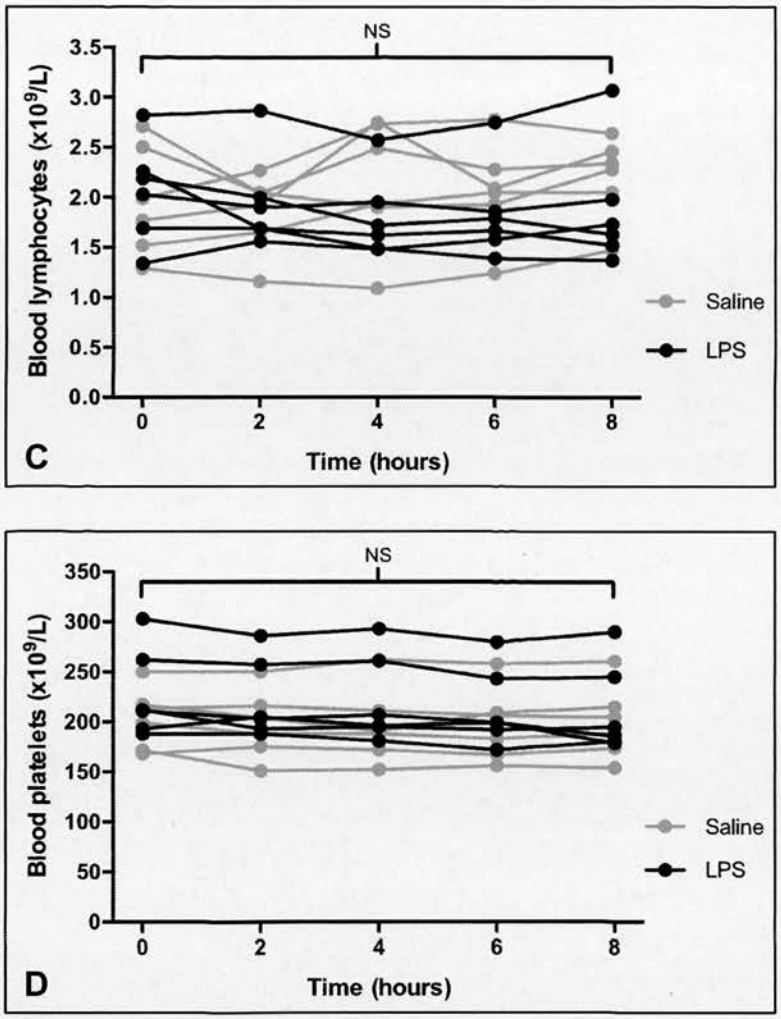


Figure 3c: Circulating blood neutrophil (A), monocyte (B), lymphocyte (C) and platelet (D) counts between baseline and 8 hours

n=6 in each group; between-groups statistical analysis was by ANCOVA, comparing the change in circulating blood cell count between baseline and 8 hours, using baseline count as covariate; ^{NS} non-significant; *** $P<0.0005$.

d) Blood platelet counts

Compared to the saline group, LPS inhalation had no significant effect upon the change in mean blood platelet count between baseline and 8 hours (228 (SD 45.1) x10^9/L at baseline and 213 (45.1) x10^9/L at 8 hours, versus 203 (30.7) x10^9/L and 200 (37.1) x10^9/L at 8 hours; P=0.07) (Figure 3c, Panel D). Mean blood platelet counts remained similar at 24 hours in both groups (226 (SD

47.3) x10^9/L in the LPS group versus 209 (31.9) x10^9/L in the saline group; *P*=0.35 for between-groups comparison with baseline).

e) Blood monocyte subsets

Flow cytometry was used to determine the proportion of each monocyte subset in blood at each time-point. Figure 3d demonstrates the gating strategy employed to identify classical, intermediate and non-classical monocytes in blood. There were only small variations in the proportions of each blood monocyte subset (as a % of total, HLA-DR$^+$ monocytes) between baseline and 24 hours, the most obvious being a non-significant trend towards a greater proportion of classical monocytes in the LPS group, compared to a lower proportion in the saline group, by 8 hours (90.6 (SD 4.0) % classical monocytes at baseline and 92.2 (2.6) % at 8 hours in the LPS group, compared to 93.2 (5.4) % at baseline and 87.7 (5.8) % at 8 hours in the saline group; *P*=0.08). These data were extrapolated, adjusting for total circulating blood monocyte counts; whereas the estimated numbers of circulating classical blood monocytes fell by 8 hours in the saline group (from 0.50 (SD 0.2) to 0.43 (0.2) x10^9/L), they rose in the LPS group (from 0.44 (0.1) to 0.65 (0.2) x10^9/L; between-groups comparison, *P*<0.01).

Each of the blood monocyte subsets was further characterised by the relative intensity of expression of a number of cell surface markers: HLA-DR, CX3CR1, CCR2, CD64, CD11b and CD62L. The pattern of expression of these markers by each monocyte subset was not altered by LPS inhalation, when compared to

saline. The results showed clear differences between the two $CD16^+$ monocyte subsets, with intermediate monocytes demonstrating stronger expression of CCR2, CD62L and CD64 than non-classical monocytes, whereas non-classical, unlike intermediate, monocytes strongly expressed CX3CR1 (Table 3D).

Table 3D: Cell surface marker expression by blood monocyte subsets

CELL SURFACE MARKER	CLASSICAL ($CD14^{++}CD16^-$)	INTERMEDIATE ($CD14^{++}CD16^+$)	NON-CLASSICAL ($CD14^+CD16^{++}$)
HLA-DR	+	++	++
CD14	++	++	+
CD16	-	+	++
CCR2	+	+	-
CX3CR1	-	+	++
CD11b	++	++	+
CD62L	++	+	-
CD64	++	++	-

The expression of cell surface antigens by each monocyte subset is defined as high (++), intermediate (+) or low/negative (+/-) according to the RFI for GMF.

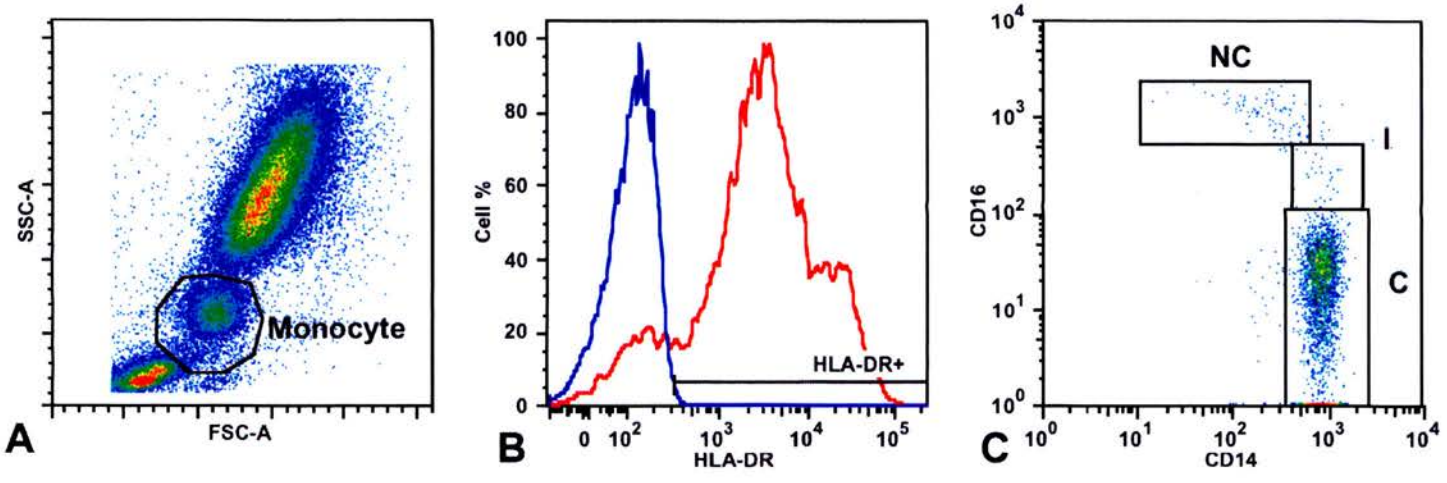


Figure 3d: Identifying blood monocyte subsets using flow cytometry

Firstly, a forward scatter-area (FSC-A) versus side scatter-area (SSC-A) plot was used to distinguish blood monocytes from neutrophils and lymphocytes by virtue of cell size and granularity (Panel A). Monocytes were further selected using HLA-DR staining thus excluding other, HLA-DR$^-$, cells (Panel B). Relative expression of CD14 and CD16 was then used to identify the three distinct monocyte subsets (Panel C). The proportion of each monocyte subset at each time-point was calculated as a % of total HLA-DR$^+$ monocytes. NC, non-classical (CD14$^+$CD16^{++}); I, intermediate (CD14^{++}CD16$^+$); C, classical (CD14^{++}CD16$^-$).

f) Cell surface marker expression by blood neutrophils

Flow cytometry was also used to identify blood neutrophils at each time-point (Figure 3e). Levels of expression of CD16, CD11b, CD62L and CD64 by blood neutrophils did not change after LPS inhalation.

g) Blood $CD4^+$ T cells and T_{reg} cells

$CD4^+$ and $CD8^+$ T cells and T_{reg} cells in blood were identified as shown in Figure 3f. The proportion of peripheral blood $CD4^+$ T cells (out of total $CD3^+$ T cells) was not influenced by LPS inhalation (mean of 54.9 (SD 4.2) % at 0 hours and 51.5 (2.2) % at 8 hours in the LPS group, versus 61.0 (5.2) % and 58.7 (7.8) % in the saline group; P=0.44). Likewise, the proportion of T_{reg} cells (out of total $CD4^+$ T cells) in peripheral blood remained stable after LPS inhalation (mean 8.1 (SD 2.4) % at 0 hours and 7.6 (1.6) % at 8 hours in the LPS group, versus 8.7 (1.2) % and 8.3 (0.7) % in the saline group; P=0.98).

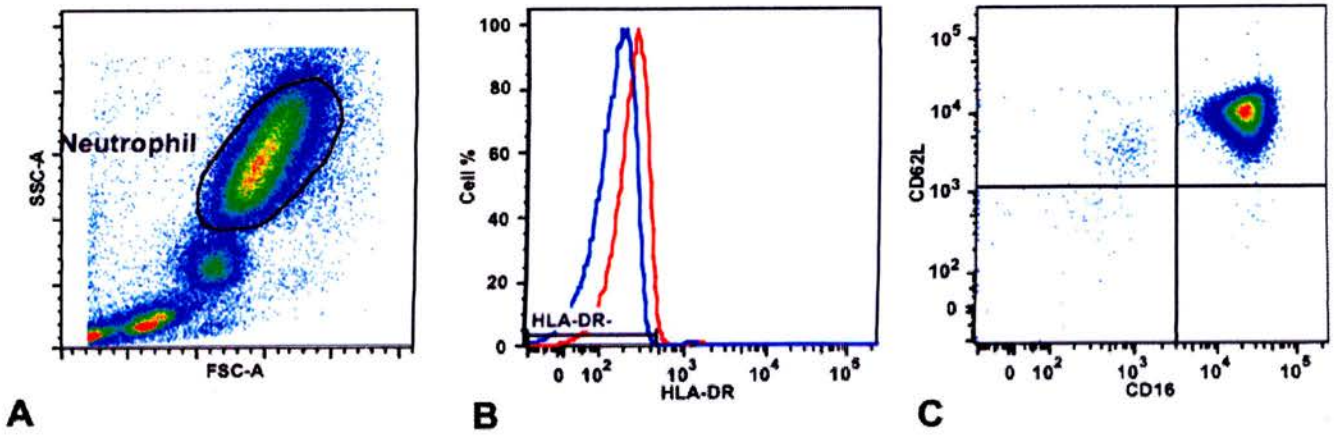


Figure 3e: Identifying blood neutrophils using flow cytometry

As before, FSC-A versus SSC-A plots were used to distinguish the neutrophil population in blood (Panel A). Neutrophils do not express HLA-DR, therefore any HLA-DR$^+$ cells were excluded from analysis (Panel B). Neutrophils were further distinguished by positive staining for CD16 and CD62L (Panel C).

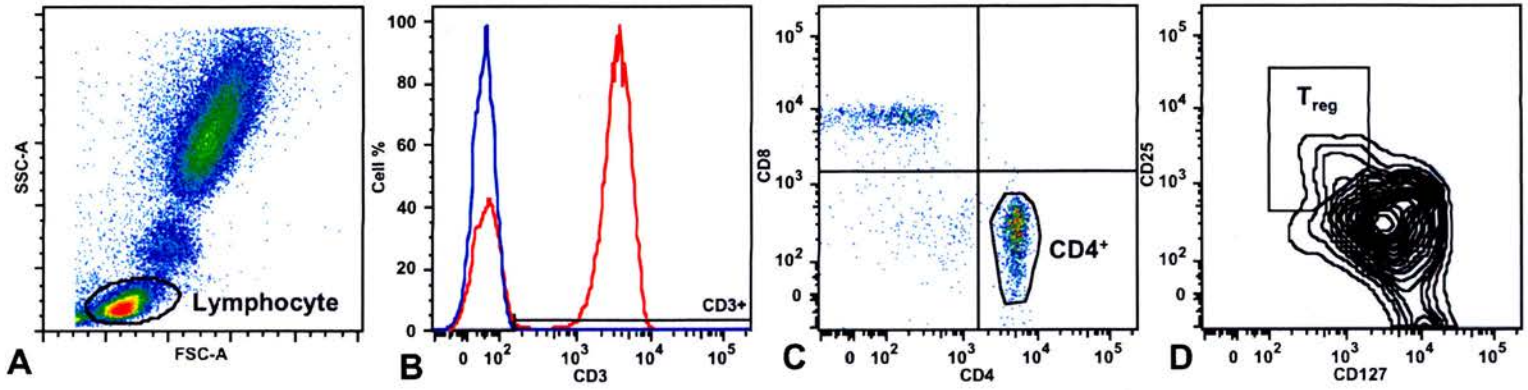


Figure 3f: Identifying T-cell subsets using flow cytometry

A FSC-A versus SSC-A plot was used to identify the blood lymphocyte population (Panel A). CD3 staining was then used to positively select ($CD3^+$) T cells and exclude other $CD3^-$ cells, including B lymphocytes (Panel B). $CD3^+$ T cells were then divided into $CD4^+$ and $CD8^+$ subsets (Panel C). Within the $CD4^+$ subset, the proportion of $CD127^-$ and $CD25^+$ T_{reg} cells could then be analysed (Fazekas de St Groth *et al.*, 2011; Shen *et al.*, 2009).

3.5 THE EFFECTS OF LPS INHALATION AS MEASURED IN BAL FLUID

a) BAL volume and total cell count

There was greater variability in the volume of BAL fluid collected from subjects in the LPS group compared to the saline group accompanied by a trend towards a lower mean volume of BAL fluid collected from the LPS group, although this did not quite reach significance (mean of 79 (SD 35) ml in the LPS group, versus 111 (12) ml in the saline group; P=0.06) (Figure 3g).

In both groups, the mean % of non-viable cells was <16% of the total automated cell count. There were a significantly greater number of total viable cells per ml of BAL fluid in the LPS group, when compared with saline placebo (mean of 3.12 (SD 1.35) x10^5/ml in the LPS group, versus 1.10 (0.35) x10^5/ml in the saline group; P=0.006) (Figure 3g).

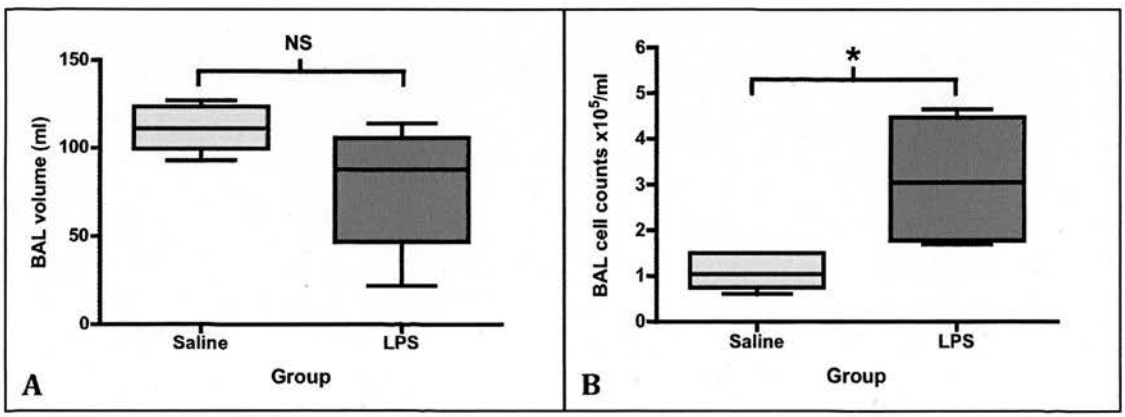


Figure 3g: Volume of BAL fluid retrieved (A) and total viable cell counts per ml (B)
Data are displayed as box (median/IQR) and whiskers (range); n=6 for the saline group and n=5 for the LPS group. Statistical analysis was by 2-sample t-test; * P<0.05.

b) BAL cell content (light microscopy)

Cytospins of BAL fluid demonstrated a prominent BAL neutrophilia in subjects who had inhaled LPS compared with very few neutrophils in the BAL fluid of subjects who had inhaled saline placebo (Image 3a).

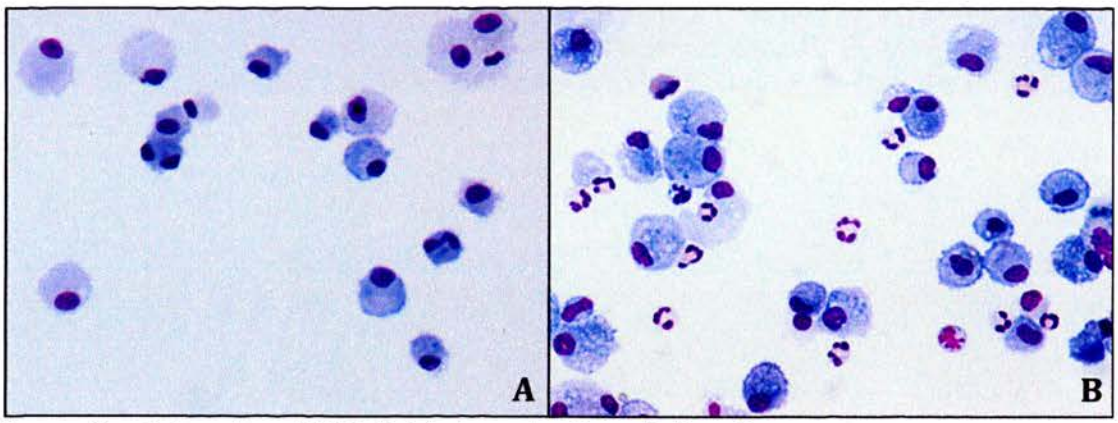


Image 3a: Cytospins of BAL fluid viewed using a light microscope
Representative images of BAL cells photographed at 400x magnification, from one subject from the saline group (Panel A) and one subject from the LPS group (Panel B).

Differential cell counts confirmed a much greater relative proportion of neutrophils (as a % of total BAL cells) and a much smaller relative proportion of AMs in the LPS group when compared to the saline group (Table 3E).

Distinguishing other types of BAL cells by simple light microscopy is widely acknowledged to be less straightforward. Indeed, it can be difficult to accurately distinguish monocytes from other leucocytes within peripheral blood (Robbins and Swirski, 2010); likewise, monocyte-like cells in BAL vary in appearance and may resemble smaller alveolar macrophages, but they can also be confused with large, activated T lymphocytes (Ward and Walters, 2001). Lymphocytes and monocyte-like cells were therefore counted as a single 'mononuclear cell'

category: the relative proportion of mononuclear cells was similar between the groups.

Table 3E: Relative proportions of each cell type present in BAL fluid

CELL TYPE	SALINE GROUP	LPS GROUP
Alveolar macrophages	97.4 (0.7)	73.8 (18.4) *
Mononuclear cells	2.0 (0.7)	3.2 (1.3) ^{NS}
Neutrophils	0.6 (0.4)	23.1 (18.4) *

Data are expressed as mean (SD); n=6 for saline group; n=5 for LPS group; statistical analysis was by 2-sample t-test; * $P<0.05$.

As could be anticipated from the greater relative proportion of neutrophils present in the BAL fluid of the LPS subjects, adjusting for total viable cell number demonstrated a significantly greater absolute number of neutrophils per ml of BAL fluid in the LPS group (mean of 172.3 (SD 187.0) $x10^3$/ml, versus 0.7 (0.4) $x10^3$/ml in the saline group; $P=0.04$) (Figure 3h).

Despite the lower relative proportion of AMs present in the BAL fluid of LPS subjects, adjusting for total viable cell number still revealed a significantly greater absolute number of AMs per ml of BAL fluid in the LPS group compared to the saline group (mean of 213.4 (SD 58.3) $x10^3$/ml in the LPS group, versus 107.2 (33.8) $x10^3$/ml in the saline group; $P=0.004$) (Figure 3h).

Adjusting for total viable cell number also revealed a much greater absolute number of MNCs per ml of BAL fluid in the LPS group (mean of 10.6 (SD 6.6) $x10^3$/ml, versus 2.3 (1.2) $x10^3$/ml in the saline group; $P=0.01$) (Figure 3h).

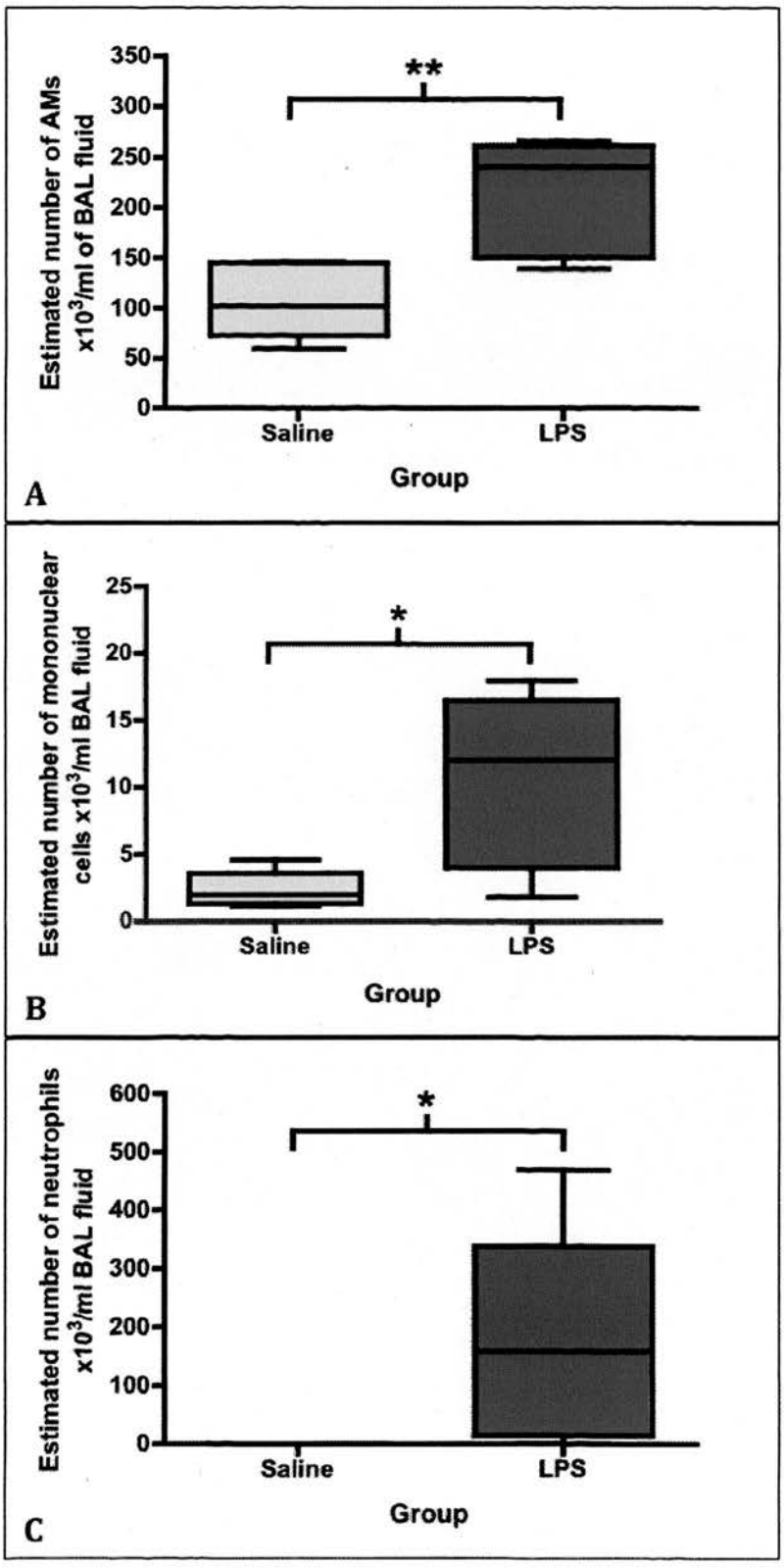


Figure 3h: Estimated numbers of (A) AMs, (B) MNCs and (C) neutrophils, per ml of BAL fluid

Data are presented as box (median/IQR) and whiskers (range); n=6 for the saline group and n=5 for the LPS group; statistical analysis was by 2-sample t-test; * $P<0.05$; ** $P<0.005$.

c) BAL cell content (flow cytometry)

Unlike light microscopy, flow cytometry enabled accurate distinction of monocyte-like cells from small AMs or large lymphocytes. The effect of LPS inhalation on the proportions of monocyte-like cells in BAL could then be determined.

Flow cytometry plots of FSC-A versus SSC-A revealed clear differences in BAL cell populations between the saline and LPS groups (Figure 3i). High numbers of neutrophils were again clearly evident in BAL from subjects within the LPS group. Furthermore, another clear population of cells distinct from neutrophils, lymphocytes or AMs was present in the FSC-A versus SSC-A plots of BAL from subjects in the LPS group (albeit this population was less clear in the flow cytometry plots for the saline group subjects). Further analysis of these cells showed that, unlike neutrophils, they were HLA-DR^{+} and that, like peripheral blood monocytes, they could be divided into subsets according to their relative expression of CD14 and CD16 (Figure 3j). These cells were termed 'pulmonary monocyte-like cells' (PMLCs).

As shown in Figure 3j, a CD14^{++}CD16^{+} subset (reminiscent of intermediate blood monocytes) was predominant in BAL from the saline subjects. These cells were termed 'resident' (rPMLC subset). In BAL from the LPS subjects, however, there was a dramatic expansion in the proportion of the CD14^{++}CD16^{-} subset (reminiscent of classical blood monocytes). These cells were therefore termed 'inducible' (iPMLC subset). Table 3F illustrates the marked difference in the relative % of rPMLC and iPMLC in the LPS and saline groups.

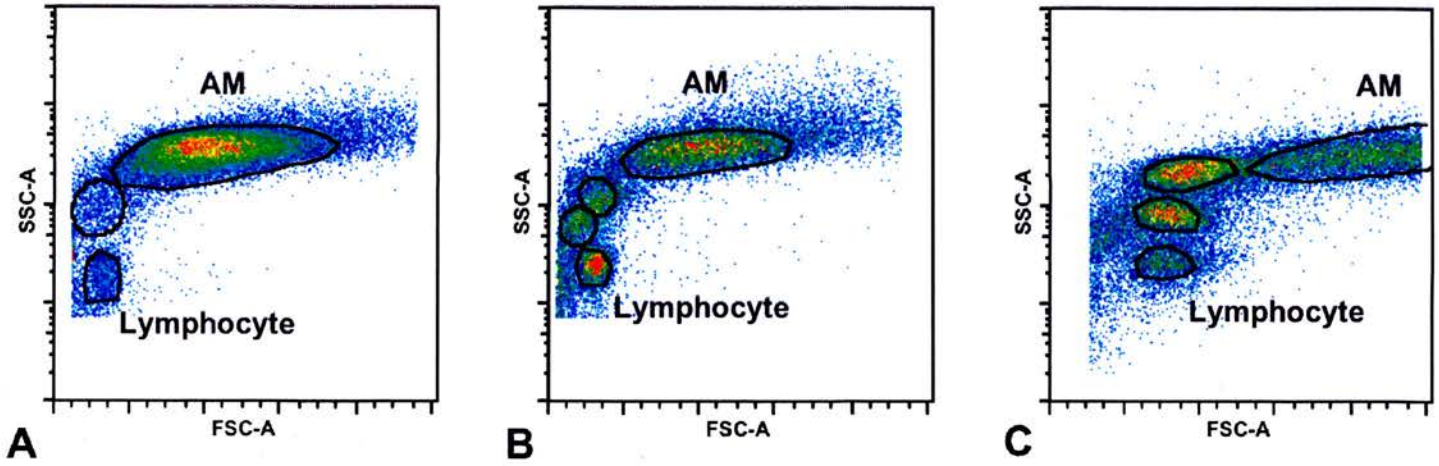


Figure 3i: Distinguishing BAL cell populations using cell size and granularity

FSC-A versus SSC-A plots from (A) a subject who had inhaled saline and (B) a subject who had inhaled LPS. Panel C shows, in closer detail, the cell populations present after inhalation of LPS. Further analysis (by cell surface antigen expression) identified the two unlabelled cell populations as neutrophils (superior) and monocyte-like cells (inferior). Neutrophils and monocyte-like cells did not form such distinct populations in the subjects who had inhaled saline, presumably due to their much lower cell numbers.

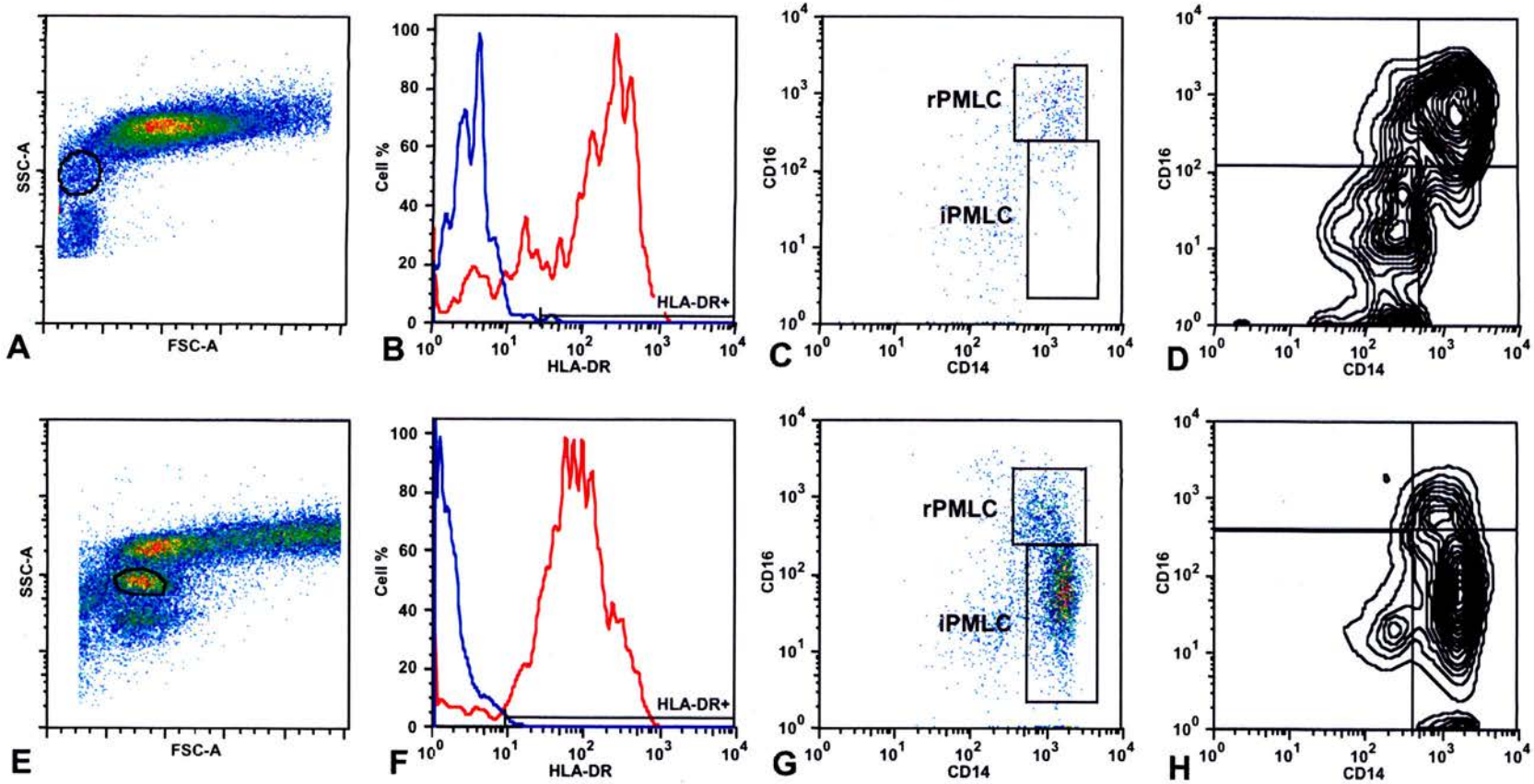


Figure 3j: Identifying PMLCs and their subsets using flow cytometry

Panels A to D are an example from a subject in the saline group; Panels E to H are an example from a subject in the LPS group. Panels A and E demonstrate the location of PMLCs on simple FSC-A versus SSC-A plots. HLA-DR$^+$ cells were selected from these gates, excluding any HLA-DR$^-$ neutrophils (Panels B and F). Two PMLC subsets were identified according to CD14 and CD16 expression; the CD14^{++}CD16$^+$ rPMLC subtype was predominant in BAL from the saline subjects (Panels C and D) and the CD14^{++}CD16$^-$ iPMLC subset was predominant in LPS subjects (Panels G and H). Panels D and H are contour plots containing the same cellular data as Panels C and G respectively.

Table 3F: The relative % of rPMLC and iPMLC in BAL fluid

PMLC SUBTYPE	SALINE GROUP	LPS GROUP	SIGNIFICANCE
rPMLC ($CD14^{++}CD16^{+}$)	65.1 (18.1)	11.6 (5.9)	** P=0.0008
iPMLC ($CD14^{++}CD16^{-}$)	34.9 (18.1)	88.4 (5.9)	

Data are presented as mean (SD) % of total PMLCs (rPMLC plus iPMLC) identified in BAL fluid; n=6 in the saline group and n=5 in the LPS group; statistical analysis was by 2-sample t-test; ** indicates a significance level of P<0.005.

Therefore, unlike light microscopy, flow cytometry allowed clear distinction of PMLCs from AMs and lymphocytes using size and granularity. Analysis of cell surface antigen expression showed that, like blood monocyte subsets, PMLC subsets could be distinguished by relative CD14 and CD16 expression, although no $CD14^{+}CD16^{++}$ PMLC subset (equivalent to non-classical monocytes) was present in BAL fluid from either group. Unlike DCs, PMLCs expressed CD11b and high levels of CD14. PMLCs also differed from blood monocytes in that they expressed CD206, a marker of monocyte maturation (Figure 3k).

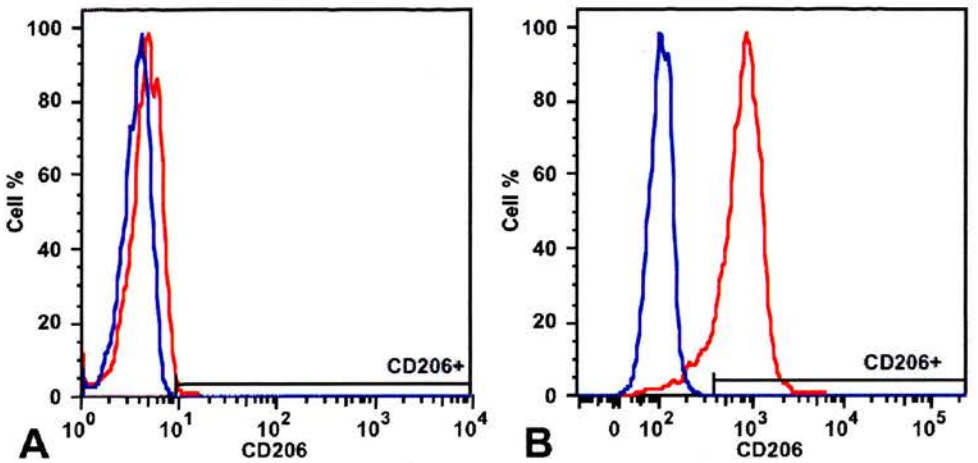


Figure 3k: CD206 expression by (A) blood monocytes and (B) PMLCs in BAL
Representative plots are shown for a subject from the saline group. Similar CD206 expression was seen in subjects from the LPS group. The blue lines represent CD206 negative (isotype/unstained) controls and the red lines represent CD206 expression by each of the cell populations.

The proportion of T_{reg} cells in BAL was calculated as for blood, as a proportion of total $CD3^+CD4^+$ cells (Figure 3f, Page 123). A significantly lower proportion of T_{reg} cells was present in BAL fluid after LPS compared to saline inhalation (3.4 (SD 0.7) % versus 6.8 (0.7) % respectively; *P*=0.01); however, total cell numbers (and estimated MNC numbers) were much higher in BAL fluid from subjects who had inhaled LPS. It is therefore unlikely that the absolute numbers of T_{reg} cells in BAL were lower after LPS inhalation.

d) Levels of protein in BAL fluid supernatant

There was much greater variability in mean protein concentration in the BAL supernatants from subjects in the LPS group and a trend towards a higher mean protein concentration, although this did not quite reach significance (median BAL protein of 0.15 [IQR 0.1 – 0.4] g/L in the LPS group, versus 0.08 [0.06 – 0.09] in the saline group, *P*=0.08 (Figure 3l).

The dilution factor was calculated for each BAL fluid sample, using plasma and BAL urea concentrations (as outlined in Methods, Chapter 2, Page 103). There was a non-significant trend towards more concentrated BAL in the LPS group (mean BAL dilution factor of 17.6 (SD 4.5), versus 27.0 (7.7) in the saline group; *P*=0.09). The BAL protein concentrations were adjusted for BAL dilution; there was no significant difference in ELF protein concentrations between the groups (median ELF protein of 2.9 [2.0 – 4.8] g/L in the LPS group compared to 2.2 [1.4 – 2.9] g/L in the saline group; *P*=0.31) (Figure 3l).

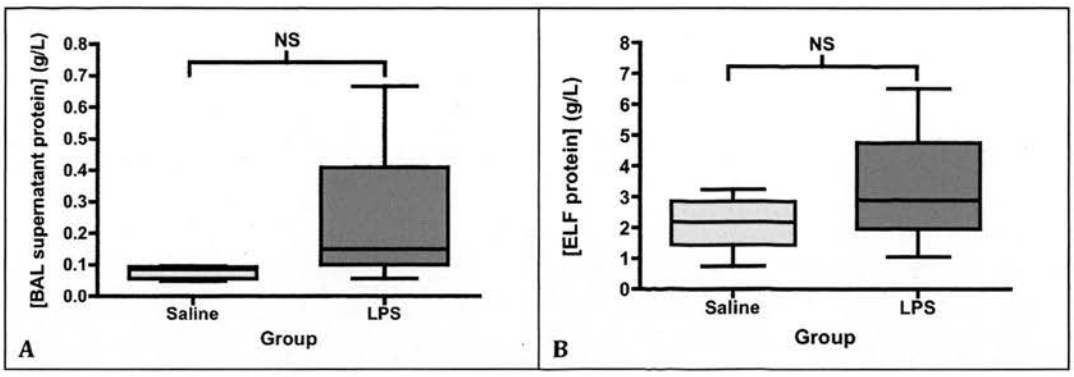


Figure 3l: Protein concentrations in BAL fluid supernatant (Panel A) and in ELF, after adjustment for BAL dilution (Panel B)

Data are displayed as box (median/IQR) and whiskers (range); n=6 for the saline group, n=5 for the LPS group. Statistical analysis was by MWU test.

e) Levels of inflammatory markers in BAL fluid supernatant

Compared to subjects who had inhaled saline, there were significantly higher concentrations of IL-6 and MCP-1 in BAL fluid supernatant from subjects who had inhaled LPS (Table 3G). There was also a trend towards higher concentrations of IL-8 in the LPS group subjects; this became significant after adjustment for BAL dilution factor. IL-10 levels were undetectable in all subjects from both groups. Levels of TNFα were undetectable in 5 subjects from the saline group, compared to 2 subjects from the LPS group.

Table 3G: Cytokines and inflammatory markers in BAL fluid supernatant

CYTOKINE/MARKER	SALINE GROUP	LPS GROUP
IL-6 (pg/ml)	56 [*x* – 94]	159 [139 – 349] **
IL-8 (pg/ml)	*x* [*x* – 51]	79 [67 – 120] †
MCP-1 (pg/ml)	*x* [*x* – 26]	81 [60 – 117] **
RAGE (pg/ml)	1980 [834 – 2890]	1690 [544 – 2780] NS
TNFα (pg/ml)	*x* [*x* – 17]	34 [*x* – 51] NS

Data are presented as median [IQR]; n=6 in the saline group; n=5 in the LPS group. Statistical analysis was by MWU test. *x* denotes a concentration below the lower limit of detection of the assay (9.4 pg/ml for IL-6, 31.3 pg/ml for IL-8, 15.6 pg/ml for MCP-1 and TNFα and 62.5 pg/ml for RAGE). ** $P<0.005$; † $P=0.03$ after correction for BAL dilution; NS non-significant.

3.6 CONCLUSIONS FROM STUDY A

a) Safety of LPS inhalation

As discussed in Chapter 1, LPS inhalation is an established model of early acute lung inflammation in healthy human volunteers. Critically, it appears to be safe, typically producing only mild and short-lived 'flu-like symptoms. Furthermore, LPS inhalation, at a dose similar to that used in this study, induces pulmonary and systemic inflammation characteristic of changes seen in early ALI (Maris, De Vos, Dessing, *et al.*, 2005; Michel *et al.*, 1997; Shyamsundar *et al.*, 2009).

Consistent with this previous literature, in this study LPS inhalation appeared safe, causing no serious adverse events. Furthermore, all symptoms attributable to LPS inhalation (including pyrexia, myalgia and cough) were mild and self-limiting. At the dose used (60μg), there was no clearly discernible effect on spirometry. Two subjects reported cough (and this may have been a side effect

of bronchoscopy) but there were no reports of chest tightness, wheeze or breathlessness suggestive of bronchospasm. One subject from the saline group reported symptoms of pyrexia, occurring several hours after BAL. Post-BAL pyrexia is a recognised phenomenon occurring in about 10-30% of cases (Ettensohn *et al.*, 1988; Krause *et al.*, 1997).

b) Efficacy of LPS inhalation as a model of inflammation

As intended, LPS inhalation was associated with clinical and biological evidence of inflammation, both systemically in blood and locally in BAL fluid. Firstly, compared to inhalation of saline placebo, inhalation of LPS caused significant rises in temperature and heart rate. Secondly, inhalation of LPS induced a sharp rise in peripheral blood neutrophil counts, peaking at 8 hours from baseline. The mean increment by 8 hours in circulating blood neutrophils in LPS subjects was 5.2 $x10^9$/L, compared to a reduction of 0.1 $x10^9$/L in the saline group subjects. There was, however, a later (albeit mostly much smaller) peak in blood neutrophil counts in the saline group, at 24 hours. This is most likely to be secondary to a direct inflammatory effect of BAL, which has been previously documented to cause a rise in blood neutrophil counts in healthy volunteers (Terashima, 2001; Essen *et al.*, 1991). Indeed, there was an extremely pronounced blood neutrophilia at 24 hours in the subject from the saline group who had reported post-BAL pyrexia, although his symptoms had already resolved and he otherwise remained well.

In terms of pulmonary inflammation, the total number of cells per ml of BAL fluid was almost three times greater in the LPS than the saline subjects, indicating LPS-induced cellular influx into the alveolar space. Both microscopic and flow cytometry analysis of BAL fluid demonstrated much larger numbers of neutrophils present after inhalation of LPS. There was also a clear trend towards higher protein levels in BAL after LPS inhalation, although this did not reach significance. Furthermore, IL-6 and MCP-1 concentrations were significantly higher in BAL from subjects who had inhaled LPS compared to saline placebo.

c) Pulmonary monocyte-like cells (PMLCs)

Using light microscopy to accurately identify monocyte-like cells in BAL proved problematic for the reasons already mentioned; however, we successfully used flow cytometry to confirm the presence of monocyte-like cells in the human alveolar space and then to further investigate their phenotype. As discussed in detail in the Introduction (Page 70), monocyte-like cells have been described in human sputum or BAL, using varying nomenclature, in several inflammatory conditions (including COPD, ILD, HIV-related lung disease, CF and ALI) and, in much smaller numbers, in healthy volunteers. However, their exact phenotype and similarity to blood monocytes has not been previously investigated.

In agreement with previous studies, monocyte-like cells in BAL were smaller and less granular than AMs. Although reminiscent of circulating monocytes, monocyte-like cells appeared phenotypically distinct from blood monocytes, in

particular with regards to their expression of CD206 (a marker of monocyte/macrophage maturation). To my knowledge, this has not been previously demonstrated and these cells were therefore termed PMLCs. Furthermore, for the first time, I have further characterised these cells according to CD14 and CD16 expression into two clear subtypes. Small numbers of PMLCs were present in the control subjects who had inhaled saline and these were mainly $CD14^{++}CD16^{+}$ ('resident', rPMLCs, reminiscent of intermediate blood monocytes). After LPS inhalation, much larger numbers of PMLCs were present and the predominant subtype was $CD14^{++}CD16^{-}$ ('inducible', iPMLCs, reminiscent of classical blood monocytes).

An accumulation of iPMLCs in the alveolar space therefore appears to be part of the LPS-induced inflammatory cascade. These cells are likely to correspond to the 'alveolar monocytes' described previously in patients with ARDS (Rosseau *et al.*, 2000) although further work is clearly required to confirm this. As described in the Introduction (Page 62), studies in $Cx_3cr1^{gfp/+}$ mice using intravital microscopy have demonstrated accumulation of the $Ly6C^{high}$ monocyte subset (equivalent to the human classical monocyte subset) in ischaemic myocardial tissue (Swirski *et al.*, 2009). In this study, I have demonstrated a rise in the estimated number of circulating classical blood monocytes after LPS inhalation. It is therefore interesting to speculate that the alveolar accumulation of iPMLCs seen in this study may represent LPS-induced transmigration of classical blood monocytes across the pulmonary endothelial-alveolar epithelial barrier.

There was no PMLC subtype akin to non-classical blood monocytes in the BAL fluid of any of the subjects. It has been argued that non-classical blood monocytes represent the more mature end of a spectrum (Gordon and Taylor, 2005; Robbins and Swirski, 2010; Wong *et al.*, 2011); perhaps the absence of a $CD14^{+}CD16^{++}$ PMLC subset indicates direct maturation of other PMLC subtypes into AMs.

d) Blood monocytes and T_{reg} cells

As discussed in the Introduction (Page 65), there is now clear evidence to support three distinct human monocyte subsets, which has lead to a new classification system where $CD16^{+}$ monocytes can be subdivided into intermediate and non-classical types (Ziegler-Heitbrock *et al.*, 2010). In line with previous research, I have shown that classical monocytes are $CD14^{++}CD16^{-}$ and that they strongly express CCR2, CD11b, CD62L and CD64. Non-classical monocytes were $CD14^{+}CD16^{++}$ and demonstrated very strong expression of CX3CR1 and HLA-DR, with weaker expression of CD11b and no expression of CCR2, CD62L or CD64. Intermediate monocytes were $CD14^{++}CD16^{+}$, also strongly expressed HLA-DR and displayed intermediate expression of CD11b, CD62L and CD64. These findings strongly support the new blood monocyte nomenclature.

Swirski's group demonstrated the presence of a large monocyte reservoir within the murine spleen, which (unlike monocytes from bone marrow) is rapidly mobilised after coronary ligation (Swirski *et al.*, 2009). It is not known if

there is a similar splenic monocyte reservoir in humans. In this study, I have shown a trend towards a rise in total blood monocyte counts 8 hours after LPS inhalation. Furthermore, when compared to saline, LPS inhalation caused a significant rise in the estimated numbers of circulating classical monocytes at 8 hours. Higher proportions of classical monocytes in human blood have been linked to greater cardiovascular risk and also to increased mortality in stroke patients (Berg *et al.*, 2012; Urra *et al.*, 2009).

Other cell types have also been implicated in the pathogenesis of ALI (Caudrillier *et al.*, 2012; D'alessio *et al.*, 2009; Looney *et al.*, 2009; Venet *et al.*, 2009). LPS inhalation did not appear to affect blood lymphocyte counts, CD4:CD8 ratio or the proportion of T_{reg} cells, when compared to saline control. Platelet levels were also unaffected by LPS inhalation. Although the proportion of BAL T_{reg} cells (as a % of $CD3^+CD4^+$ cells) dropped after LPS inhalation, the dramatic rise in total BAL cell numbers in BAL could mean that absolute numbers of T_{reg} cells in BAL rise after LPS. T_{reg} cells are thought to be involved in the resolution phase of the inflammatory cascade and there is preliminary evidence that their numbers increase in BAL fluid from patients with ALI (D'Alessio *et al.*, 2009).

3.7 LIMITATIONS OF STUDY A

a) Subject demographics and sample size

The groups were well matched in terms of baseline characteristics; however, the low mean age and gender restriction may have limited the relevance of my results, especially as gender may influence the response to IV LPS (Coyle *et al.*, 2006; Van Eijk *et al.*, 2007). All three of my studies were restricted to subjects between the ages of 18 and 40, in order to limit the risk of undetected health problems that could compromise subject safety and data interpretation. All the studies were restricted to male subjects to maintain a consistent approach (ethical approval for Study C depended upon removing any potential radiation exposure from PET-CT scanning to an unborn foetus).

The main limitation of this study was the small sample size of six subjects in each group. This limited statistical power, in particular with regards to whether inhaled LPS causes a definite rise in circulating blood monocyte count (a finding that would support the early mobilisation of a human splenic monocyte reservoir in response to inflammation, equivalent to that seen in mice). The estimated numbers of classical monocytes did appear to rise after LPS; however, this (and the effect upon numbers of other monocyte subsets) requires further confirmation using a larger sample size.

Small sample size is also likely to explain why the trends toward higher BAL protein and IL-8 levels after LPS inhalation did not reach significance, as they

have done in previous studies (Maris, De Vos, Dessing, *et al.*, 2005; Shyamsundar *et al.*, 2009).

b) Cell identification by light microscopy

Another limitation of this study relates to the difficulties in accurately distinguishing monocyte-like cells by light microscopy, as they can resemble large lymphocytes or small AMs (Ward and Walters, 2001). We therefore aimed to categorise monocyte-like cells along with lymphocytes as 'mononuclear cells'. As expected, there was a rise in the number of MNCs present in BAL after LPS inhalation. Although the overall proportion of AMs in BAL fell after LPS inhalation, when corrected for total cell number there was actually a rise in the number of AMs per ml of BAL after LPS inhalation; this may reflect partial misclassification of monocyte-like cells as small AMs rather than an actual proliferation of macrophages within the alveolar space. Flow cytometry allowed us to more accurately classify BAL cell types, although it did not provide us with absolute cell numbers (estimated numbers of each cell type were instead derived from the cell proportion multiplied by the total, automated cell count).

c) BAL as a means of analysing alveolar inflammation

Analysing BAL fluid provides extremely useful information in the drive to understand the pathophysiology of lung inflammation; however, it does have some inherent limitations. The concentration of ELF within a sample of BAL fluid is dependent upon the sample dilution. Although the same volume of lavage fluid was used in each subject, this cannot guarantee that an equal BAL

return volume or concentration is obtained (Baughman, 1997). Indeed, there was a trend towards lower and more variable BAL fluid volumes in the subjects who had inhaled LPS (which may, from simple observation, have been due to an increased tendency for the LPS subjects to cough during bronchoscopy). Adjusting for BAL dilution using the urea method (Rennard *et al.*, 1986) did not have a great impact upon the results, indicating that variable BAL dilution was not likely to be a major issue. Another potential drawback of BAL is that only one lobar segment is sampled, which may mean that BAL fluid is not representative of overall alveolar inflammation throughout both lungs. Furthermore BAL only samples the alveolar space, not the pulmonary interstitium, and is therefore potentially not representative of 'whole' lung inflammation (Reynolds, 2000; Schultheis and Bassett, 1991). The process of BAL itself can induce inflammation (as seen by the rise in blood neutrophil counts in the saline subjects at 24 hours). It is also difficult to ethically justify performing BAL at more than one time-point in healthy subjects. Many previous LPS inhalation studies have used induced sputum instead of BAL to analyse alveolar inflammation (for example Michel *et al.*, 1997; Michel *et al.*, 1997; Thorn and Rylander, 1998). Although obtaining induced sputum is a less invasive procedure than bronchoscopy and can be more easily repeated (i.e. data can be easily obtained from more than one time-point), sputum is less likely than BAL to contain cells from the alveolar space and much more likely to contain a significant number of cells originating from the proximal bronchial tree (Alexis *et al.*, 2001; Antoniou *et al.*, 2005; Lensmar *et al.*, 1998).

Despite the limitations above, BAL remains a valuable research tool; this study used a consistent, standardised method for both bronchoscopy and BAL to try to minimise variability (Haslam and Baughman, 1999).

d) The LPS inhalation model

The LPS model of lung inflammation has some limitations. It activates the innate immune system transiently, whereas true pathological stimuli are likely to be more prolonged. ALI is a heterogeneous condition in that it has many different causes; inhalation of LPS models only pulmonary (as opposed to non-pulmonary) causes of ALI. Distribution of LPS throughout the lungs may be uneven when it is given by inhalation, although careful administration should minimise this (Brand *et al.*, 2000; Häkkinen *et al.*, 1999; Popa, 2001). Some studies of LPS-induced lung inflammation have used bronchial (segmental) instillation to enable more controlled deposition while also providing an intra-subject control (using saline lavage of a lobar segment of the opposite lung), although this procedure requires bronchoscopy at baseline and is therefore a much more invasive technique to use in healthy volunteers (Chen *et al.*, 2006; Hoogerwerf *et al.*, 2008; O'Grady *et al.*, 2001). Despite its limitations, LPS inhalation as performed in this study provides a controlled, consistent model of early pulmonary inflammation that is safe and acceptable to healthy subjects.

3.8 SUMMARY OF STUDY A

In conclusion, Study A fulfilled its primary objective as a preliminary test of the safety and efficacy of LPS inhalation prior to commencing the RCT. Furthermore, I was able to use flow cytometry to demonstrate the presence of PMLCs in human BAL fluid which were distinct from peripheral blood monocytes and that could be clearly divided into two subtypes. Finally, I showed an LPS-induced accumulation of the iPMLC subtype that may represent transmigration of classical monocytes from blood and that may be integral to the development of the alveolar neutrophilia typical of ALI.

Having completed Study A, I performed a second preliminary study in a separate group of healthy subjects, this time examining the use of leukapheresis for blood monocyte depletion.

CHAPTER 4: RESULTS OF STUDY B

4.1 OVERVIEW

This chapter describes the results of Study B, which looked at MNC depletion by leukapheresis in a group of six healthy subjects.

The main aims of this study were:

- To ensure the **safety and efficacy of leukapheresis** as a means of monocyte depletion in healthy subjects
- To examine the **extent of monocyte depletion** achieved by leukapheresis
- To determine whether leukapheresis itself has any **direct systemic or pulmonary inflammatory sequelae**, in particular whether it affects blood neutrophil counts or BAL neutrophil, protein and cytokine content.

This chapter will first outline subject recruitment and demographic data, symptoms, adverse events and the effects of leukapheresis on clinical parameters. It will then provide data on the extent and nature of cell depletion by MNC leukapheresis in relation to the FBC data. The effects of leukapheresis on the cellular, protein and inflammatory marker content of BAL fluid will also be presented.

4.2 SUBJECTS

a) Subject recruitment

Six subjects were recruited into the study as illustrated in the CONSORT diagram (Figure 4a). Blood and BAL data are complete for all six subjects; however, antibody staining of the MNC collection for flow cytometry was only successful in three of the six subjects.

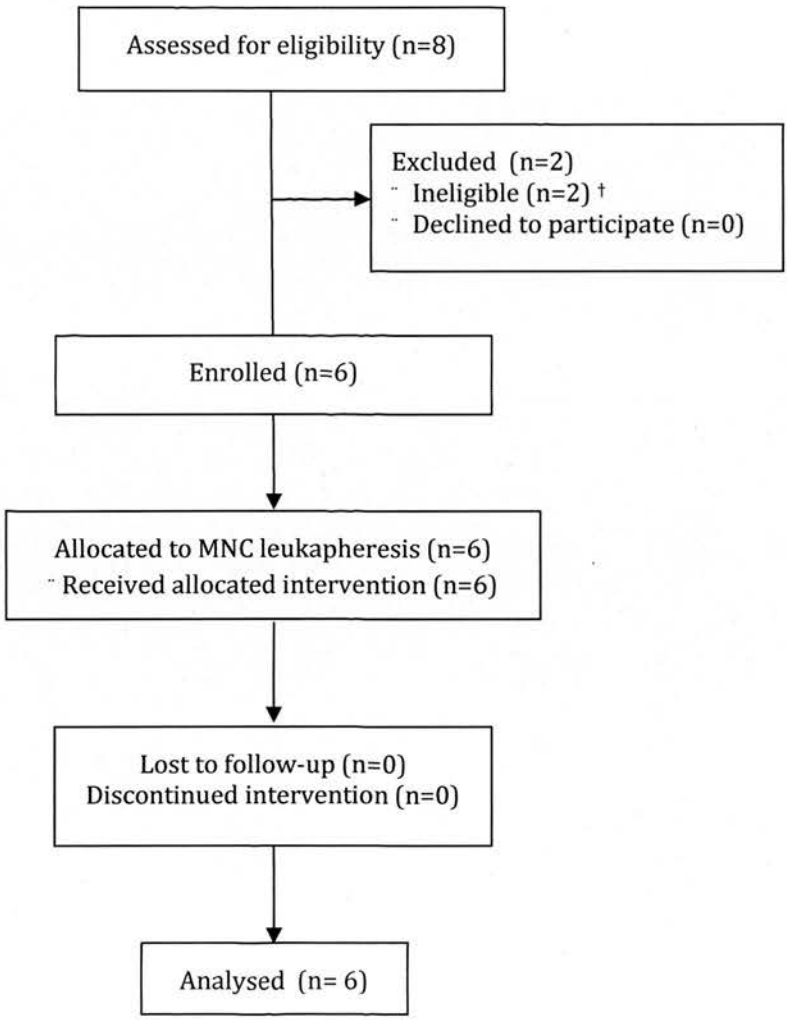


Figure 4a: CONSORT diagram illustrating volunteer recruitment, enrolment and follow-up during Study B

† Reasons for exclusion: elevated bilirubin (n=1); current smoker (n=1).

b) Subject demographics

The baseline characteristics of the subjects are outlined in Table 4A. These were broadly similar to the characteristics of the Study A subjects (see Table 3A, Page 112), although mean age was slightly lower and mean spirometry values were slightly higher.

Table 4A: Study B subject demographics at baseline

BASELINE CHARACTERISTIC	MNC LEUKAPHERESIS GROUP
Age (years)	20.5 (1.0)
Ethnicity	6/6 Caucasian
Height (metres)	1.83 (0.05)
Weight (kg)	75.7 (4.9)
FEV_1 (litres)	5.01 (0.53)
FVC (litres)	5.77 (0.56)
Temperature (°C)	35.9 (0.5)
Heart rate ($minute^{-1}$)	66 (9)
Oxygen saturations (%)	98 (1)

Data are presented as mean (SD); n=6.

The subjects' mean estimated TBV was 5.335 (SD 0.293) L. All six subjects had 4 TBVs processed during leukapheresis (mean of 4.1, SD 0.1), equivalent to a mean processed blood volume of 21.6 (SD 1.2) L. The mean duration of leukapheresis was just over 6 hours, at 370 (SD 29) minutes. There was little variability in the amount of ACD-A used for anticoagulation during the procedure (mean 1701 (SD 190) ml).

c) Symptoms and adverse events

Four subjects reported a mild sore throat following bronchoscopy, all symptoms resolving within 24 hours of discharge. Three volunteers described facial and/or digital paraesthesiae during their leukapheresis procedure consistent with citrate-induced hypocalcaemia, all of which resolved with treatment. The first subject required 6 doses of oral calcium in total; the other 5 volunteers were therefore given a combination of oral and IV calcium supplementation during leukapheresis, which effectively minimised symptomatic hypocalcaemia.

4.3 THE EFFECTS OF LEUKAPHERESIS ON CLINICAL PARAMETERS

There was a small but significant rise in mean temperature during the course of leukapheresis (mean temperature of 35.9 (SD 0.5) °C at baseline compared to 36.6 (0.1) °C after leukapheresis; P=0.02) (Table 4B). Other clinical parameters were unaffected by MNC leukapheresis.

Table 4B: Clinical parameters at baseline and after leukapheresis

CLINICAL PARAMETER	AT BASELINE	AFTER LEUKAPHERESIS
Temperature (°C)	35.9 (0.5)	36.6 (0.1) *
HR (minute^{-1})	72 (14)	66 (9) ^{NS}
sBP (mmHg)	123 (28)	119 (32) ^{NS}
RR (minute $^{-1}$)	14 (13 - 14)	15 (14 - 16) ^{NS}
SaO_2 (%)	98 (1)	99 (1) ^{NS}

Data are presented as mean (SD) or median [IQR]; n=6. Statistical analysis by paired t-test or Wilcoxon signed rank test; * P<0.05.

4.4 CELL RETRIEVAL AND YIELD FROM LEUKAPHERESIS

a) Cell retrieval

The MNC collections retrieved by leukapheresis varied little in volume (mean of 0.345 (SD 0.029) litres). Table 4C lists the numbers of each cell type collected by MNC leukapheresis, derived from automated cell counts performed on a representative sample. As anticipated, leukapheresis removed large numbers of monocytes, lymphocytes and platelets but very few neutrophils, basophils or eosinophils. Indeed, a small number of neutrophils (0.98 $x10^9$) were only sequestered in the MNC collection of one of the six subjects.

Table 4C: Cell counts in the mononuclear cell collections

CELL TYPE	TOTAL CELL NUMBER ($x10^9$)
RBCs	165 (37.9)
Platelets	986 (137)
Total WBCs	30.9 (4.27)
Lymphocytes	23.5 (4.47)
Monocytes	5.57 [4.67 – 10.6]
Basophils	0.19 (0.13)
Neutrophils	0 [0 – 0.49]
Eosinophils	0 [0 – 0.03]

Data are presented as mean (SD) or median [IQR]; n=6.

b) Monocyte yield

There was a very strong correlation between the total number of monocytes depleted (and therefore present in the MNC collection at the end of leukapheresis) and the respective total circulating monocyte count at baseline

(r=0.98 [$CI_{0.95}$ 0.82, 1.00]) (Figure 4b, Panel A). The median yield of monocytes from leukapheresis (the total number of monocytes in the MNC collection divided by the total number of baseline circulating blood monocytes) was 2.3 [IQR 2.2 – 3.1].

c) Lymphocyte yield

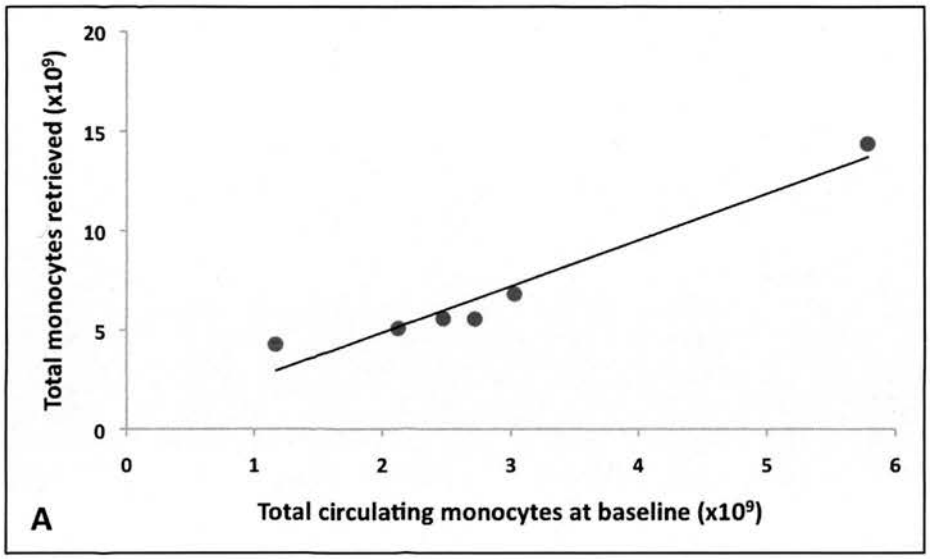
Panel B in Figure 4b shows the correlation between the total number of lymphocytes depleted by leukapheresis and the respective total circulating lymphocyte count at baseline (r=0.83 [$CI_{0.95}$ 0.04, 0.98]. The mean yield of lymphocytes from MNC leukapheresis was 2.1 (SD 0.32).

d) Platelet yield

There was also a correlation between the total number of platelets depleted and the respective total circulating platelet count at baseline (r=0.87 [$CI_{0.95}$ 0.19, 0.99]) (Figure 4b, Panel C). Mean platelet yield from leukapheresis was 0.85 (SD 0.09).

e) RBC yield

There was no clear correlation between the total number of RBCs depleted by leukapheresis and the respective total circulating RBC count at baseline (r=-0.34 [$CI_{0.95}$ -0.90, 0.65]) (Figure 4b, Panel D). Mean RBC yield was 0.006 (SD 0.002).


Total monocytes retrieved (x10⁹)
20
15
10
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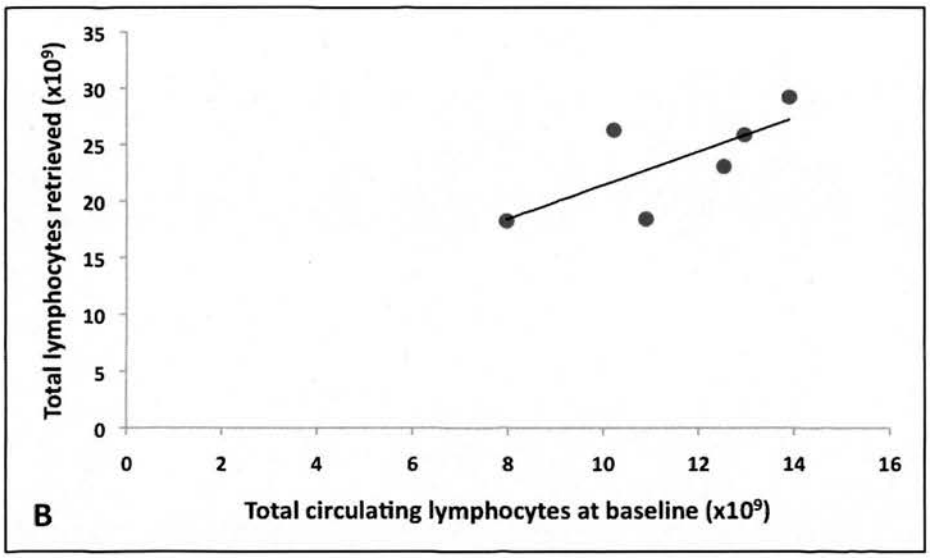
1
2
3
4
5
6
A
Total circulating monocytes at baseline (x10⁹)


Total lymphocytes retrieved (x10⁹)
35
30
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0
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12
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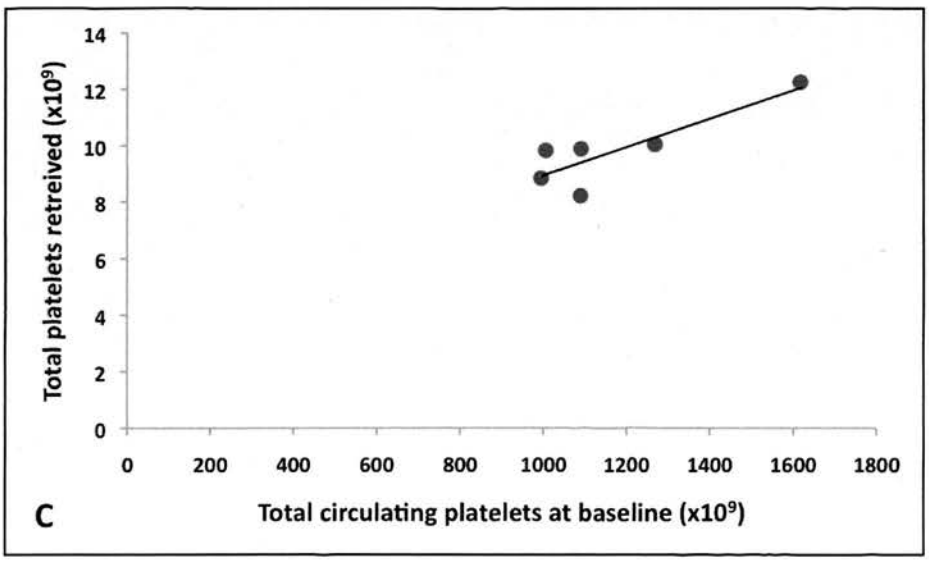
16
B
Total circulating lymphocytes at baseline (x10⁹)


Total platelets retreived (x10⁹)
14
12
10
8
6
4
2
0
0
200
400
600
800
1000
1200
1400
1600
1800
C
Total circulating platelets at baseline (x10⁹)

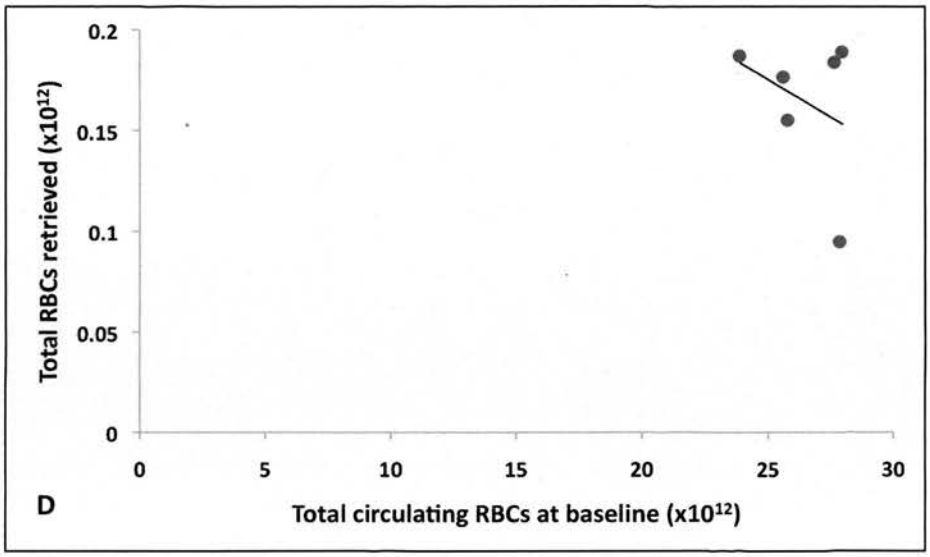


Figure 4b: Correlations between (A) baseline blood monocyte, (B) lymphocyte, (C) platelet and (D) RBC counts and the corresponding MNC collection cell counts
n=6; statistical analysis by Pearson's test.

4.5 THE EFFECTS OF LEUKAPHERESIS AS MEASURED IN BLOOD

a) Circulating blood neutrophil counts

Mean circulating blood neutrophil count remained stable at 8 hours (3.25 (SD 2.00) $x10^9$/L, compared to a baseline mean of 3.13 (1.71) $x10^9$/L; *P*=0.65) (Figure 4c, Panel A). There was, however, a significant rise in mean circulating blood neutrophil count by 24 hours when compared to baseline (5.38 (SD 1.79) $x10^9$/L; *P*=0.03). As before, the sixth subject had obviously higher circulating blood neutrophil counts at all time-points, including at baseline.

b) Circulating blood monocyte counts

There was a significant fall in median circulating blood monocyte count by 8 hours (to 0.32 [IQR 0.21 – 0.65] $x10^9$/L, from a baseline of 0.50 [0.30 – 0.86]

$x10^9$/L; *P*=0.03) (Figure 4c, Panel B). Median circulating blood monocyte count at 24 hours had recovered to a level just above baseline (0.59 [IQR 0.53 – 0.87] $x10^9$/L; *P*=0.22). The sixth subject had higher counts at all time-points, including at baseline, although the pattern over time was otherwise similar.

c) Circulating blood lymphocyte counts

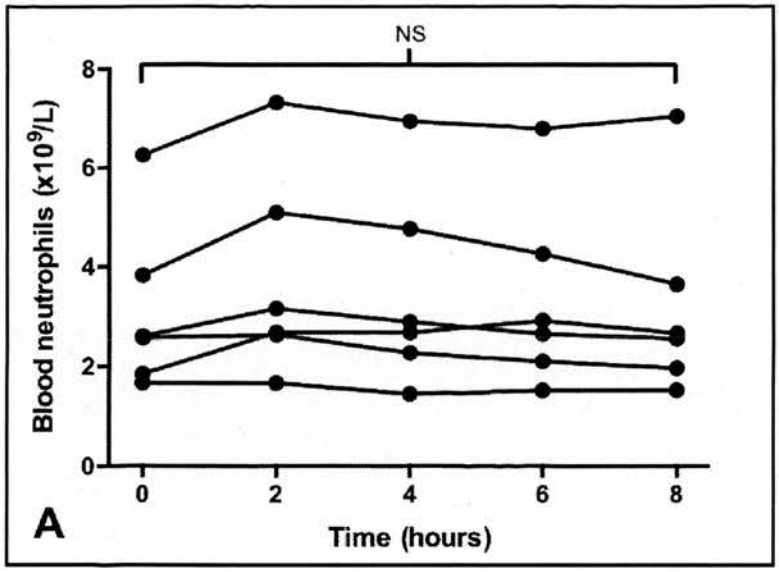
There was a significant fall in mean circulating blood lymphocyte count by 8 hours (to 1.61 (SD 0.32) $x10^9$/L, compared to a baseline of 2.14 (0.41) $x10^9$/L; *P*=0.0004) (Figure 4c, Panel C). This fall in mean circulating blood lymphocyte count persisted at 24 hours (with a mean of 1.60 (SD 0.29) $x10^9$/L; *P*=0.02).

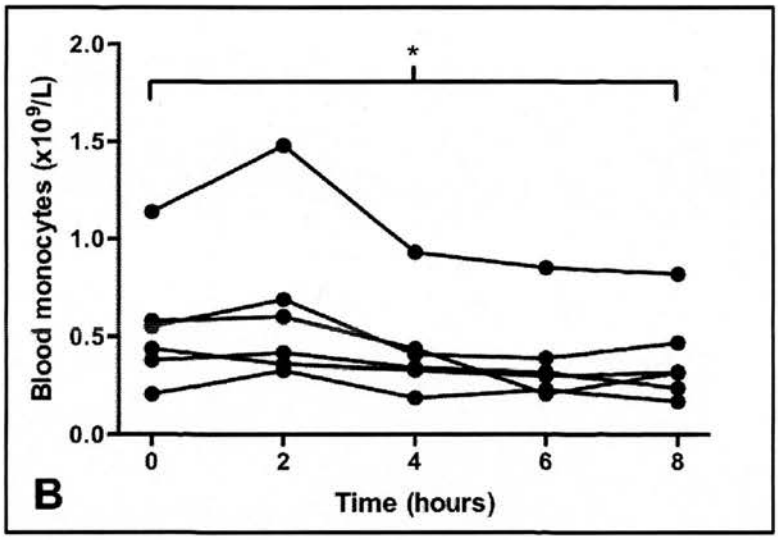
d) Circulating blood platelet counts

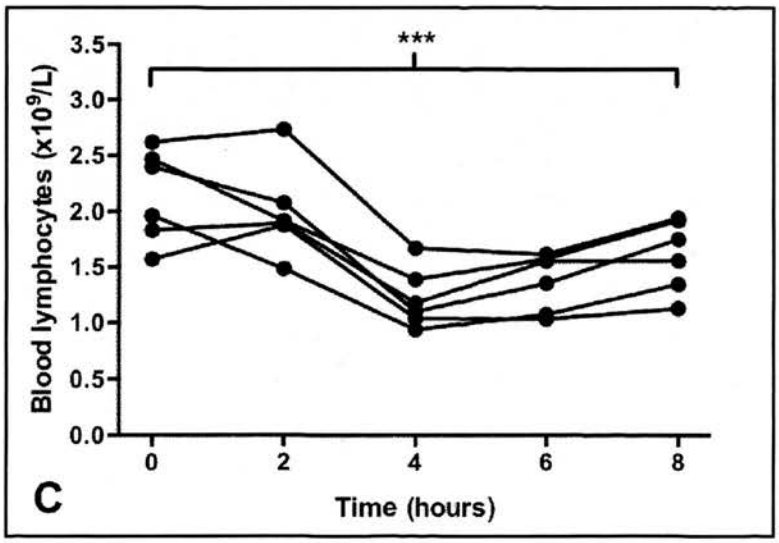
There was also a significant fall in median circulating blood platelet count by 8 hours (to 93 [IQR 76 – 133] $x10^9$/L, from a baseline of 196 [188 – 285] $x10^9$/L; *P*=0.03) (Figure 4c, Panel D). This fall in median circulating blood platelet count persisted at 24 hours (103 [IQR 88 – 144] $x10^9$/L; *P*=0.03).

e) Circulating RBC counts

Mean circulating RBC count fell significantly by 8 hours, from a baseline of 4.96 (SD 0.08) $x10^{12}$/L to 4.61 (0.07) $x10^{12}$/L (*P*=0.0004) (Figure 4c, Panel E). Mean circulating RBC count had, however, recovered to baseline levels by 24 hours (mean of 4.88 (SD 0.16) $x10^{12}$/L; *P*=0.29).


NS
Blood neutrophils (x10⁹/L)
8
6
4
2
0
0
2
4
6
8
A
Time (hours)


*
Blood monocytes (x10⁹/L)
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
B
Time (hours)


***
Blood lymphocytes (x10⁹/L)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
C
Time (hours)

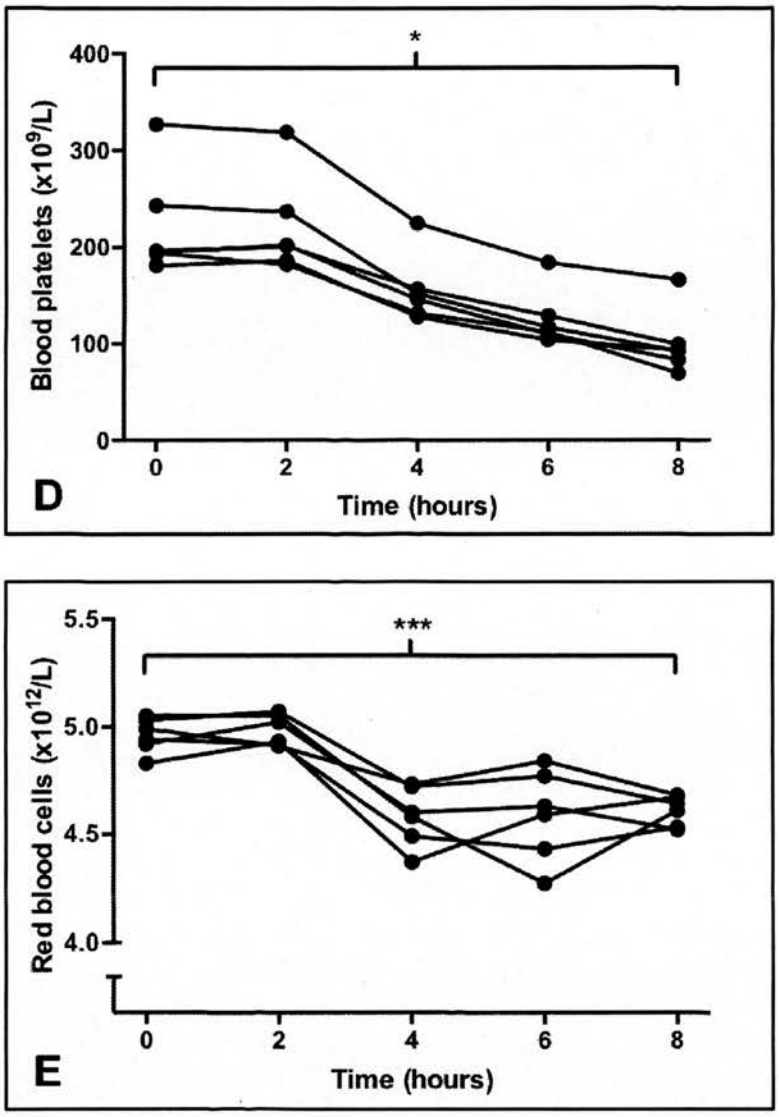


Figure 4c: Circulating blood neutrophil (A), monocyte (B), lymphocyte (C), platelet (D) and RBC (E) counts between baseline and 8 hours

MNC leukapheresis began at 2 hours and ended at around 8 hours. n=6; statistical analysis of the change in blood cell counts between baseline and 8 hours was by paired t-test (for neutrophils, lymphocytes and RBCs) or by Wilcoxon signed rank test (for monocytes and platelets); * $P<0.05$; *** $P<0.005$.

f) Blood monocyte subsets

As for Study A, flow cytometry was used to calculate the relative proportions of each blood monocyte subset, as a proportion of total HLA-DR$^+$ monocytes, at each time-point (see Figure 3d, Page 120). The mean proportion of classical

monocytes at baseline was similar to the proportions seen in the Study A subjects at baseline (93.0 (SD 1.7) %, compared to 93.2 (5.4) % in the saline group and 90.6 (4.0) % in the LPS group), but rose significantly by the completion of leukapheresis to 98.8 (1.0) % (P=0.01). After adjusting for the fall in total circulating blood monocyte count after leukapheresis, the estimated numbers of classical blood monocytes demonstrated a less marked reduction (from 0.47 [0.38 – 0.81] to 0.32 [0.28 – 0.56] x10^9/L at 8 hours) compared to intermediate and non-classical monocytes. The proportion of classical monocytes remained elevated at 98.7 (0.9) % at 24 hours (P=0.01), with the estimated numbers of classical monocytes recovering to (and beyond) baseline levels (0.59 [0.53 – 0.85] x10^9/L).

4.6 MONOCYTE SUBSETS RETRIEVED BY LEUKAPHERESIS

Flow cytometry was also used to identify monocytes and their subsets within three of the MNC collections retrieved by leukapheresis, using a gating strategy similar to that used for blood (Figure 4d). The mean proportion of classical monocytes within the MNC collections was 84.1 (SD 9.2) %; this compared to 93.1 (1.0) % in peripheral blood at baseline in the same subjects. Intermediate monocytes made up a mean of 8.0 (SD 6.2) % of monocytes within the MNC collections, compared to 1.7 (0.2) % in baseline peripheral blood. The mean proportion of non-classical monocytes within the MNC collections was 7.9 (SD 4.3) %, compared to 5.2 (1.9) % at baseline in peripheral blood.

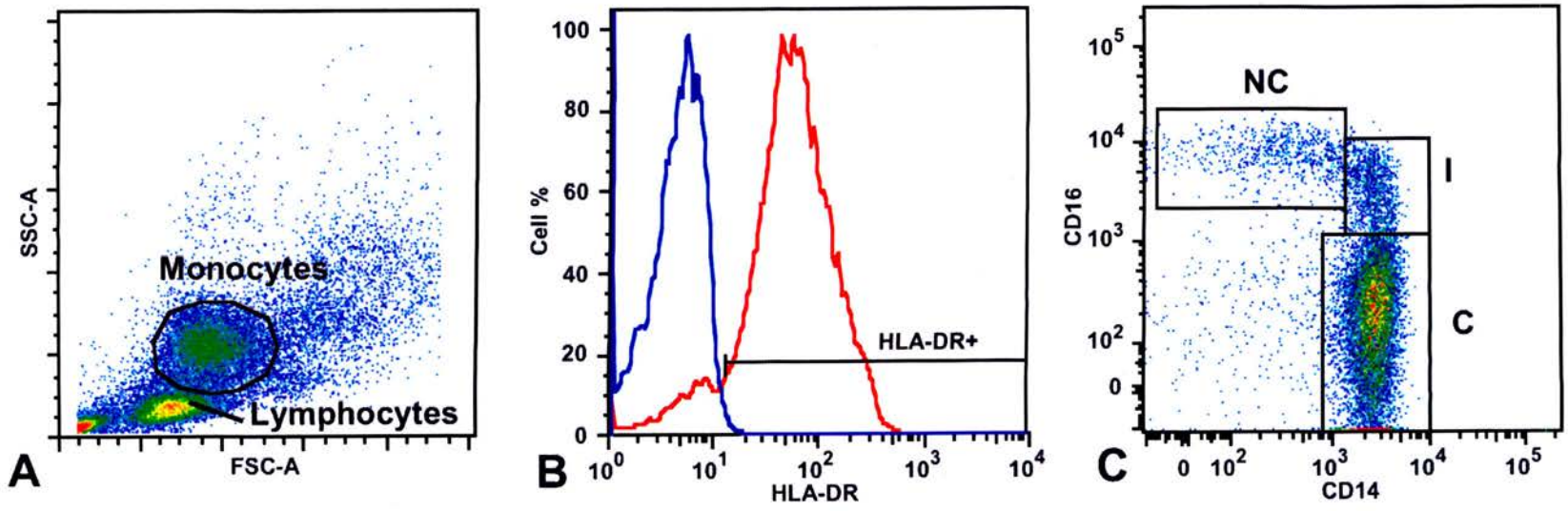


Figure 4d: Identifying blood monocyte subsets within the MNC collection obtained by leukapheresis

Example flow plots are shown from one representative subject (of n=3). A FSC-A versus SSC-A plot was used to gate on the monocyte population (Panel A), excluding most lymphocytes and debris. Monocytes were further selected using HLA-DR staining, thus excluding other (HLA-DR negative) cells (Panel B). Relative expression of CD14 and CD16 was then used to identify the three monocyte subsets (Panel C). NC, non-classical ($CD14^{+}CD16^{++}$); I, intermediate ($CD14^{++}CD16^{+}$); C, classical ($CD14^{++}CD16^{-}$). The proportion of each monocyte subset at each time-point was expressed as a percentage of total $HLA\text{-}DR^{+}$ monocytes.

4.7 THE EFFECTS OF LEUKAPHERESIS AS MEASURED IN BAL FLUID

a) BAL volume and total cell count

The mean volume of BAL fluid collected was 72 (SD 44) ml (slightly lower than the mean of 111 (12) ml retrieved from the Study A saline subjects). The median total viable cell count in BAL was 2.18 [IQR 1.38 – 4.80] x 10^5/ml, higher than the mean of 1.10 (SD 0.35) x10^5/ml in the Study A saline subjects. The mean proportion of non-viable cells was <20% of the total automated cell count.

b) BAL cell content (light microscopy)

As discussed in the previous chapter, accurately identifying cell types in BAL fluid by light microscopy proved difficult. Lymphocytes and monocyte-like cells were therefore again counted under a single MNC category. The median proportion of AMs in BAL fluid was 96.7 [IQR 89.7 – 98.5], compared to 97.4 [96.7 – 98.1] in the saline group from Study A. Neutrophils made up a median proportion of 1.7 [IQR 0.2 – 8.2] %, compared to 0.7 [0.2 – 0.9] % in the saline group from Study A. Interestingly, the sixth subject (who had higher circulating neutrophil and monocyte counts at all time-points, including at baseline, and who was the only subject to demonstrate neutrophil sequestration during leukapheresis) also demonstrated an unexpectedly high BAL neutrophil content of 10.3%. Finally, the median proportion of mononuclear cells in BAL fluid was 1.7 [IQR 0.8 – 2.7] %, compared to 2.1 [1.4 – 2.7] % in the saline group from Study A.

After adjusting for total cell counts, there was a median of 214.8 [IQR 132.7 – 431.5] $x10^3$ AMs/ml of BAL fluid, a median of 3.4 [0.1 – 38.3] $x10^3$ neutrophils/ml and a median of 3.0 [1.4 – 10.2] $x10^3$ MNCs/ml.

c) BAL cell content (flow cytometry)

As in Study A, flow cytometry was used to identify AMs, lymphocytes, neutrophils, PMLCs and PMLC subtypes in BAL fluid. The cell populations visible on FSC versus SSC plots (Figure 4e) appeared similar to those of the saline group subjects from Study A (see Figure 3i, Page 129). However, in contrast to the saline group subjects from Study A, the inducible ($CD14^{++}CD16^{-}$) subtype of PMLCs was more prominent, although not to the extent seen in the LPS group subjects from Study A (see Figure 3j, Page 130). A mean of 56.7 (SD 8.7) % were of the iPMLC subtype (reminiscent of classical monocytes), compared to 34.9 (18.1) % in the Study A saline subjects. A mean of 43.3 (8.7) % were of the rPMLC subtype ($CD14^{++}CD16^{+}$, reminiscent of intermediate monocytes), compared to 65.1 (18.1) % in the Study A saline subjects. As in Study A, there was no clearly discernible population of $CD14^{+}CD16^{++}$ PMLCs (akin to non-classical monocytes) in the BAL fluid of any of the six subjects.

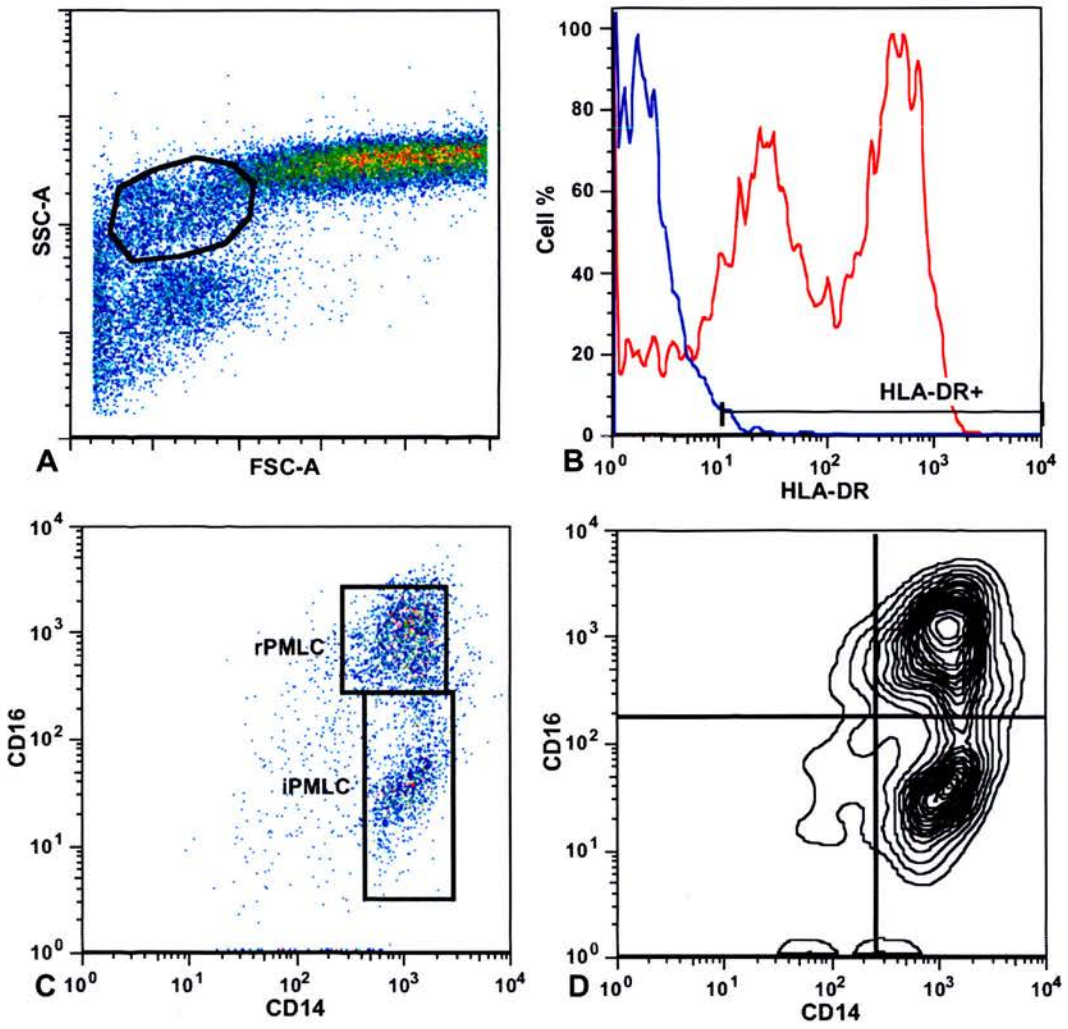


Figure 4e: Identifying PMLCs in BAL fluid after MNC leukapheresis
Example flow plots are shown from one representative subject (of n=6). Panel A demonstrates the gate drawn to incorporate PMLCs and neutrophils on a FSC-A versus SSC-A plot. HLA-DR$^+$ cells were then positively selected, in order to exclude HLA-DR$^-$ neutrophils (Panel B). The two PMLC subsets (rPMLCs and iPMLCs) were distinguishable using relative CD14 and CD16 expression (Panel C). Panel D is a contour plot containing the same cellular data as Panel C.

d) Levels of protein and inflammatory markers in BAL fluid supernatant

Mean BAL protein content was 0.11 (SD 0.06) g/L (very similar to the median BAL protein content of 0.08 [IQR 0.06 – 0.09] g/L in the Study A saline subjects). Concentrations of inflammatory markers in BAL fluid supernatant are presented in Table 4D. As in the Study A saline subjects, BAL concentrations of TNFα were

either undetectable or very low. Levels of RAGE were also very similar to those in the saline subjects from Study A, although levels of IL-6 and IL-8 tended to be a little higher (for comparison with Study A, see Table 3G on Page 134).

Table 4D: Concentrations of inflammatory markers in BAL fluid supernatant

INFLAMMATORY MARKER	CONCENTRATION IN BAL (pg/ml)
IL-6	115 [49.5 – 167]
IL-8	51.0 [41.0 – 138.0]
MCP-1	24.0 [*x* – 72.0]
RAGE	2300 [1610 – 2680]
TNFα	*x* [*x* – 19.0]

Data are presented as median [IQR]; n=6. *x* denotes a concentration below the lower limit of detection of the assay (9.4 pg/ml for IL-6, 31.3 pg/ml for IL-8, 15.6 pg/ml for MCP-1 and TNFα and 62.5 pg/ml for RAGE).

4.8 CONCLUSIONS FROM STUDY B

a) Safety of leukapheresis

Leukapheresis of four TBVs was safely used to deplete MNCs from circulating blood in six healthy subjects, with no serious adverse events. As expected, the procedure was well tolerated; the most common symptoms reported (facial and digital paraesthesiae) were attributable to citrate-induced hypocalcaemia. The prevalence of symptoms relating to citrate toxicity (50%) was higher in our study than is reported in the literature (Donmez *et al.*, 2011; McLeod *et al.*, 1999; Reik *et al.*, 1997; Strauss, 1996), presumably due to the large volumes of blood processed.

The first volunteer had recurrent citrate-related symptoms requiring six doses of oral calcium supplements. Previous studies of plasmapheresis and PBSC collection have demonstrated effective control of citrate toxicity using IV calcium infusions (Bolan, 2002; Weinstein, 1996). Therefore from the second volunteer onwards, irrespective of symptoms, we commenced an IV calcium infusion midway through leukapheresis which, together with oral calcium supplements as needed, lead to prompt resolution of any paraesthesiae.

Crucially, none of the subjects developed any sign of infection as a consequence of MNC leukapheresis. As discussed, one subject demonstrated evidence of a possible subclinical infection, with higher blood monocyte counts and higher blood and BAL neutrophil counts; however, he also had higher blood monocyte and neutrophil counts at baseline. Despite this, he did not become unwell during or after leukapheresis.

Despite the large volumes of blood processed and the long duration of the procedures, none of the subjects reported feeling light-headed. Vasovagal events have been estimated to occur in up to 5% of subjects undergoing leukapheresis (Strauss, 1996).

b) Efficacy of leukapheresis for monocyte depletion

I processed a greater volume of blood (around 21 L) than is described in most of the previous leukapheresis literature, and retrieved a median of 5.6×10^9 monocytes. This further supports previous evidence of a strong correlation between processed blood volume and the number of monocytes retrieved by

leukapheresis (Nguyen *et al.*, 2002; Svensson *et al.*, 2005; Wolf *et al.*, 2005). For example, three previous studies in healthy subjects processing 4.5 L, 9 L and 10 L blood have demonstrated retrieval of 0.98 $x10^9$ (Wolf *et al.*, 2005), 2.55 $x10^9$ (Svensson *et al.*, 2005) and 2.09 $x10^9$ (Strasser *et al.*, 2003) monocytes respectively and a study in cancer patients demonstrated retrieval of 3.2 $x10^9$ monocytes after processing 11.7 L blood (Nguyen *et al.*, 2002).

In line with this previous literature, I also found a very strong correlation between the baseline circulating blood monocyte count and the number of monocytes retrieved by leukapheresis. Furthermore, in my study, the number of monocytes collected by leukapheresis was actually 2.3 times higher than the number of circulating blood monocytes at baseline. This phenomenon has been well documented, indicating monocyte repopulation of circulating blood during leukapheresis (thus obtaining a 'collection efficiency' of >100%, or a 'recruitment factor' >1) (Alteri and Leonard, 1983; Bojanic *et al.*, 2011; Strasser *et al.*, 2005; Svensson *et al.*, 2005; Wolf *et al.*, 2005).

c) Monocyte recruitment during leukapheresis

The extent of monocyte recruitment into circulating blood is demonstrated by comparison of the pre- and post-leukapheresis blood monocyte counts. In this study, the mean blood monocyte count at 8 hours was only 35% lower than that at baseline, despite removal during leukapheresis of more than twice the number of baseline circulating monocytes. Such a discrepancy is typical, with many studies noting a much smaller than anticipated fall in circulating blood

monocyte counts (Alteri and Leonard, 1983; Knudsen *et al.*, 2001; Nguyen *et al.*, 2002; Strasser *et al.*, 2005; Wolf *et al.*, 2005). This, together with the overshoot seen in blood monocyte counts at 24 hours in this study (which were 20% higher than baseline), provides further evidence for active large-scale recruitment of monocytes into circulating blood during and after leukapheresis.

The exact origin of these newly recruited monocytes remains unclear. Monocytes present in blood immediately post-leukapheresis do appear temporarily immature in terms of chemotactic responses, perhaps indicating early release from bone marrow (Alteri and Leonard, 1983). The rapidity and extent of monocyte release is, however, surprising. More recent studies have confirmed the existence in mice of both a marginating monocyte pool (Auffray *et al.*, 2007) and a reservoir of monocytes in the red pulp of the spleen (Swirski *et al.*, 2009). Furthermore, Swirski's group demonstrated rapid release of splenic monocytes into the circulation in response to an inflammatory stimulus (myocardial ischaemia), with no change in bone marrow monocyte content. If splenic and/or marginating monocytes also exist in humans, their release into circulating blood may explain the rapid monocyte repopulation seen during leukapheresis.

In this study, I saw a small but significant rise in the proportion of classical monocytes present in blood after leukapheresis. After adjusting for the overall reduction in total blood monocyte count by 8 hours, this equated to a less marked fall by 8 hours, and a quicker recovery by 24 hours, in the numbers of

classical monocytes compared to intermediate and non-classical monocytes after MNC leukapheresis.

Cell depletion by leukapheresis is dependent upon cell density; classical monocytes are larger and more dense than the $CD16^+$ subsets (Geissmann *et al.*, 2008; Robbins and Swirski, 2010) and it is therefore possible that $CD16^+$ monocytes are more likely to be removed by leukapheresis, thus explaining the apparent rise in the proportion of classical monocytes in post-leukapheresis blood. Analysis of monocyte subset proportions within the MNC collections was based upon a very small sample size (of n=3); comparing these numbers with those in circulating blood at baseline does suggest possible greater depletion of non-classical and intermediate than classical monocytes although a larger study is clearly required to confirm these findings. A previous study in patients with malignancy undergoing peripheral blood stem cell collection demonstrated preferential depletion of $CD16^+$ (intermediate and non-classical) monocyte subsets; however, the effects of malignancy and pre-mobilisation with chemotherapy and G-CSF are unclear (Tanaka *et al.*, 1999).

It is also possible that monocytes newly recruited to the blood during leukapheresis may be more likely to be classical in phenotype. If this is the case, it may better explain the greater proportion (and lower fall in number) of classical monocytes by 8 hours and the overshoot at 24 hours. In mice, initial repopulation of blood after monocyte depletion by liposomal clodronate is predominantly by $Ly6C^{high}$ monocytes, thought to be equivalent to classical monocytes in humans (Sunderkötter *et al.*, 2004). However, both subtypes of

murine monocyte appear to be released from spleen after an inflammatory stimulus (Swirski *et al.*, 2009).

The significance of a greater proportion of circulating classical monocytes is unknown. Although originally labelled as the 'pro-inflammatory' subset, classical monocytes have also been shown to release anti-inflammatory mediators such as IL-10 (Robbins and Swirski, 2010; Soehnlein and Lindbom, 2010). Higher levels of circulating classical monocytes do, however, appear to predict future risk of cardiovascular events (Berg *et al.*, 2012) and also correlate with greater mortality and poorer outcome in stroke patients (Urra *et al.*, 2009).

d) Pulmonary effects of leukapheresis

Filtration leukapheresis has been shown to induce pulmonary neutrophil sequestration and impair gas exchange but, unlike centrifugation leukapheresis, also causes systemic neutropenia (Hammerschmidt *et al.*, 1978). There have not, to my knowledge, been any previous studies on the potential pulmonary effects of centrifugation leukapheresis.

None of the six subjects reported any respiratory symptoms potentially attributable to leukapheresis. Total cell counts in BAL after leukapheresis were slightly higher than anticipated when compared to the saline control group in Study A, but not to the extent seen after LPS inhalation in Study A. Alveolar neutrophil influx was evident in only one subject, who had higher baseline circulating neutrophil and monocyte counts in blood, but who was not clinically

unwell during the study. Otherwise, mean proportions of AMs, MNCs and neutrophils in BAL were within the ranges normally seen in healthy subjects.

As in Study A, flow cytometry was used to analyse the proportions of PMLCs in BAL fluid. After leukapheresis, there was a clear population of the $CD14^{++}CD16^{+}$ rPMLCs (reminiscent of intermediate blood monocytes) in BAL, similar to the saline control subjects from Study A. Compared to these historical controls, however, there was also a distinct population of $CD14^{++}CD16^{-}$ iPMLCs (akin to classical blood monocytes) present in BAL fluid, although not as prominent as that seen within the BAL fluid of the LPS group from Study A.

This finding raises the question of whether leukapheresis induces monocyte influx across the pulmonary vascular endothelium and into the alveolar space. This study has clearly shown that removal of monocytes by leukapheresis induces rapid and extensive monocyte recruitment into peripheral blood, with an overshoot beyond baseline in the 24-hour blood monocyte count. Classical monocytes have been shown to rapidly migrate to sites of inflammation (Swirski *et al.*, 2009); it is possible that the rise in the proportion of classical blood monocytes after leukapheresis may be responsible for the apparent influx of iPMLCs to the alveolar space, without requiring a local (pulmonary) inflammatory stimulus. Perhaps relevant to this is the increased risk of pulmonary leucostasis seen in patients with the monocytic subtype of acute myeloid leukaemia (Azoulay *et al.*, 2003).

Levels of protein in BAL were also slightly higher than in the historical control subjects from Study A, as were concentrations of IL-6, IL-8 and MCP-1, although none to the extent seen after LPS inhalation in Study A. It is therefore possible that MNC leukapheresis has a subclinical effect within the pulmonary compartment. Other studies have also demonstrated subtle changes in the pulmonary compartment (without discernible alveolar neutrophilia) after systemic or non-local inflammatory stimuli. For example, IV LPS in healthy human subjects increases lung permeability and the levels of pro-inflammatory cytokines in BAL fluid (Boujoukos *et al.*, 1993; Suffredini *et al.*, 1992). In mice, intra-peritoneal LPS has been shown to cause monocyte influx into the alveolar space (Steinmüller *et al.*, 2006).

4.9 LIMITATIONS OF STUDY B

a) Small sample size

This was only a small study, with six subjects undergoing MNC leukapheresis. This sample number was sufficient for the main purpose of verifying the safety and efficacy of the procedure for depleting blood monocytes from healthy human subjects. However, the small sample size does limit the conclusions I can otherwise draw from this study, in particular with regards to monocyte populations within the MNC collections (where n=3). A larger study is therefore needed to establish whether MNC leukapheresis causes preferential depletion of $CD16^+$ monocyte subtypes, whether it causes a rise in the proportion of classical

monocytes in circulating blood and whether it does cause a subclinical effect in the pulmonary compartment (in terms of a rise in BAL cell count, protein and inflammatory cytokine levels and in the number of iPMLCs).

b) Non-depletion effects of leukapheresis

Study B was not designed to include a control group, as this was not required to test safety and efficacy. I was able to compare the data from BAL fluid with the historical control group from Study A, who had inhaled saline at baseline but were otherwise very similar to the Study B subjects in terms of baseline characteristics and blood and BAL sampling undertaken during the study.

The lack of a true control group within Study B meant, however, that I was unable to ascertain whether MNC leukapheresis has any immune-modulating effects beyond cellular depletion. Leukapheresis involves the extra-corporeal diversion of blood followed by anticoagulation and centrifugation, with separation of different cell layers. Although previous studies have frequently employed a 'sham' leukapheresis procedure (identical except for actual cell collection) in their control arms, little is known about whether sham leukapheresis can itself activate cells or exert any other kind of inflammatory effect. Crucially, monocyte chemotaxis appears unaffected by sham centrifugation leukapheresis (Alteri and Leonard, 1983), but it remains possible that leukapheresis can have other effects beyond cellular depletion that may be responsible for the effects seen in BAL during this study.

The subjects in this study did demonstrate a small rise in body temperature similar to the historical control subjects from Study A. There is no mention in the literature of an effect of leukapheresis on body temperature and this is likely to simply reflect natural diurnal variation (Lowry, 2003; Martinez-Nicolas *et al.*, 2011). The rise in blood neutrophil counts at 24 hours was also similar to the historical control subjects in Study A and can be attributed to the inflammatory effect of BAL rather than any delayed effect of leukapheresis (Terashima, 2001; Essen *et al.*, 1991).

c) Extent of monocyte depletion

This study employed large-volume leukapheresis, processing on average more than 20 L of blood over approximately 6 hours. Large numbers of circulating monocytes were actively removed during the course of MNC leukapheresis. Despite this, substantial monocyte recruitment during leukapheresis meant that there was a much smaller drop in circulating blood monocyte counts than would be otherwise anticipated. Processing even larger volumes of blood during leukapheresis would be less acceptable, particularly in healthy subjects, due to increasing duration and risk of citrate toxicity. Furthermore, processing larger volumes during leukapheresis is unlikely to achieve a much greater fall in circulating blood monocyte count, for the reasons already described.

d) Depletion of other cells

MNC leukapheresis is not selective for monocytes and does deplete other cells from circulating blood. Leukapheresis removed a large number of lymphocytes,

again demonstrating a collection efficiency of >100%. Blood lymphocyte counts dropped by an average of only 24%, indicating that lymphocytes are also recruited into circulating blood during leukapheresis, as has been suggested previously (Strasser *et al.*, 2005). However, the effect was probably not so sustained as for monocytes, as blood lymphocyte counts remained low at 24 hours.

Large numbers of platelets were also removed from circulating blood, although fewer than were present in circulating blood at baseline. Platelet yield was 0.85 but mean blood platelet count fell by 53% during the course of leukapheresis. A relatively low level of recruitment of platelets has also been reported in other studies (Nguyen *et al.*, 2002; Strasser *et al.*, 2005).

In comparison, neutrophils were only depleted from one subject, possibly due to his higher circulating neutrophil count at baseline. MNC leukapheresis therefore had no significant impact upon blood neutrophil counts, which was a key consideration when designing the studies due to the primary end-point chosen for the RCT (see Chapter 2, Page 107). Indeed, the MNC program on the COBE Spectra™ apheresis machine was ideal for our purposes as it enables rapid blood volume processing and minimises neutrophil and RBC contamination of the resultant cell collection (Bellavita *et al.*, 1997; Wolf *et al.*, 2005).

The number of RBCs depleted during leukapheresis was extremely small relative to their number in circulating blood; the transient fall in mean

circulating RBC count seen post-procedure is likely to reflect dilution due to the brief increase in circulating volume (caused by ACD-A infusion).

4.10 SUMMARY OF STUDY B

This study, for the first time, looked at the potential pulmonary effects of leukapheresis; although leukapheresis appeared to increase the proportion of iPMLCs in the lung, there was no significant neutrophil influx and no evidence of a clinically important pro-inflammatory effect in the alveolar space. A larger, controlled study is required to more closely analyse how blood monocyte subsets are affected by leukapheresis and also whether leukapheresis has pro-inflammatory effects beyond cellular depletion.

In summary, MNC centrifugation leukapheresis was successfully used to retrieve large numbers of circulating monocytes from the blood of six healthy volunteers. Although leukapheresis did not achieve sustained monocyte depletion on the scale achieved in mice by other methods (Dhaliwal *et al.*, 2012; Maus *et al.*, 2002), it does provide a safe and readily available method of monocyte depletion in humans, with evidence of benefit in other inflammatory disorders.

Having established, with Studies A and B, the safety and efficacy of both LPS inhalation for modelling human lung inflammation and MNC leukapheresis for the depletion of circulating blood mononuclear cells, I was then able to proceed to Study C, an RCT of MNC depletion in experimental human lung inflammation.

CHAPTER 5: RESULTS OF STUDY C

5.1 OVERVIEW

This chapter describes the results of Study C, a randomised double-blind sham-controlled trial of 30 healthy subjects (n=15 in each group).

The main aim of the study was to assess whether peripheral blood MNC depletion by leukapheresis could ameliorate the systemic and pulmonary inflammation induced by LPS inhalation. The pre-defined end-points were:

PRIMARY

- The **rise in blood neutrophil count by 8 hours** (from baseline)

SECONDARY

- Change in **oxygen saturations**
- Changes in **plasma cytokines/cell injury markers and CRP** (markers of systemic inflammation)
- Changes in **BAL fluid neutrophils, protein and cytokines/cell injury markers** (markers of alveolar inflammation)
- Changes in **mean K_i and SUV derived from [^{18}F]FDG PET imaging** (markers of global lung inflammation)

The secondary aim of this study was to further define the phenotype and function of PMLCs in BAL, using flow cytometry to analyse the expression of proliferation and maturation markers and using flow sorting to isolate BAL cell populations for culture and assays of cytokine release.

This chapter describes subject recruitment and demographic data, symptoms and adverse events and the effects of MNC depletion on clinical parameters, blood, BAL and PET markers of systemic and pulmonary inflammation. I will then present additional data on the phenotype and function of PMLCs in BAL.

5.2 SUBJECT RECRUITMENT

Thirty subjects were recruited into the study and randomised into either the depletion or the sham group as shown in the CONSORT diagram (Figure 5a).

Most data are complete for all 30 subjects; 'n' numbers are stated for each analysis. One subject from the sham group did not undergo bronchoscopy, as per the pre-defined safety criteria, because his FEV_1 had fallen by >10% below his baseline level.

PET-CT scans were only performed in a sub-group of 20 subjects (n=10 in each group). There were several reasons for this, including an initial delay with scanner installation and calibration. After the first two PET-CT scans had been performed (on the 5th and 6th subjects), further scans were temporarily put on hold while an ethics amendment, including a revision of estimated total radiation dose, received approval. One planned PET-CT scan was cancelled on the day due to scanner malfunction (before any [^{18}F]FDG was administered).

Staining of blood or BAL cells for flow cytometry was not performed for 2 subjects from the sham group and 1 subject from the depletion group due to

absence of laboratory staff. Further analysis of PMLC phenotype and function was performed on smaller sub-groups, as described in the relevant section.

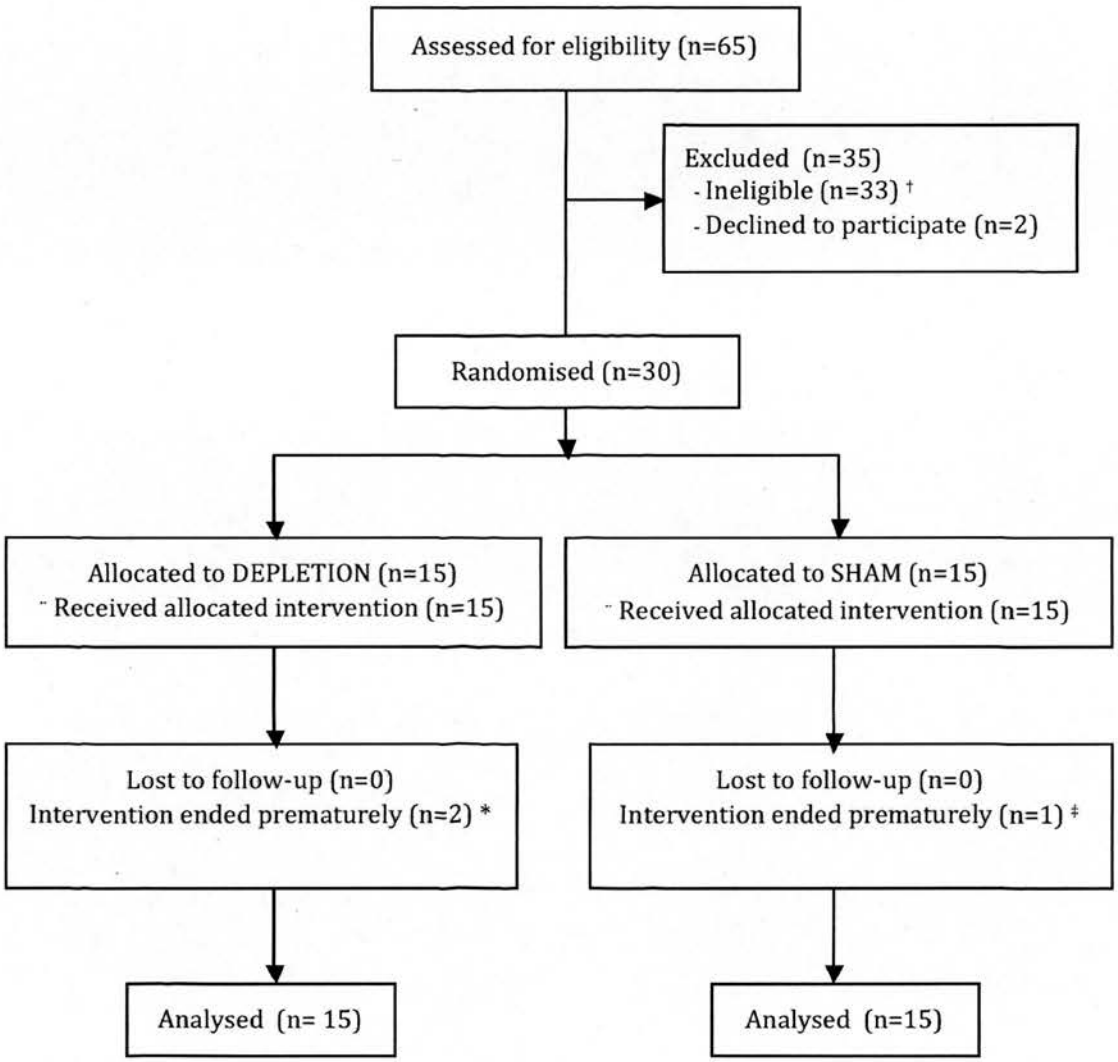


Figure 5a: CONSORT diagram illustrating subject recruitment, randomisation, intervention and follow-up during Study C

† Reasons for exclusion: abnormal total/differential WBC (n=25), bilirubin (n=7) or ALT (n=4), eGFR or urea (n=2) and platelets (n=1); FEV_1/FVC <70% (n=1); recent formal blood donation (n=1).

Three subjects had fewer than 3.5 TBVs processed: * vasovagal event (n=1, 2.4 TBVs processed in 285 minutes); high estimated blood volume/time constraint (n=1, 3.3 TBVs processed in 390 minutes); ‡ recurrent venospasm limiting flow rate during leukapheresis (n=1, 2.8 TBVs processed in 395 minutes).

5.3 SUBJECT DEMOGRAPHICS

Subjects in the two groups were well matched (Table 5A). Baseline characteristics were also similar to those of the subjects from the preliminary studies (see Table 3A, Page 112 and Table 4A, Page 147).

Table 5A: Subject demographics at baseline

BASELINE CHARACTERISTICS	SHAM GROUP	DEPLETION GROUP
Age (years)	22 [21-24]	23 [21-24]
Height (metres)	1.80 (0.05)	1.79 (0.04)
Weight (kilograms)	80.5 (12.5)	81.3 (10.3)
FEV_1 (litres)	4.78 (0.51)	4.72 (0.54)
FVC (litres)	5.75 (0.60)	5.53 (0.64)
Temperature (°C)	36.2 (0.5)	36.4 (0.5)
HR ($minute^{-1}$)	74 (9)	72 (9)
sBP (mmHg)	130 (10)	134 (15)
SaO_2 (%)	98 (1)	98 (1)

Data are presented as mean (SD) or median [IQR]; n=15 each group.

All subjects from both groups successfully completed inhalation challenge with approximately 60μg of LPS. As intended, sham leukapheresis was almost identical to the true procedure, incorporating extra-corporeal blood diversion, anticoagulation and centrifugation but omitting actual cell collection (i.e. whole blood was returned to the subject). Double blinding was maintained throughout all procedures as described in Chapter 2 (Page 87) and emergency un-blinding was not required in any subject.

The groups did not vary significantly in estimated blood volume at baseline, the number of TBVs processed, the duration of leukapheresis, the amount of ACD-A anticoagulation required or the amount of oral and/or IV calcium supplementation used (Table 5B).

Table 5B: Variables associated with the leukapheresis/sham procedures

VARIABLE	SHAM GROUP	DEPLETION GROUP
Estimated blood volume at baseline (L)	5.34 (0.54)	5.38 (0.39) [NS]
TBVs processed	4.0 [3.9 – 4.0]	4.0 [3.9 – 4.0] [NS]
Duration (hours)	5.8 (0.5)	5.7 (0.5) [NS]
Amount of ACD-A (L)	1.52 [1.44 – 1.63]	1.57 [1.51 – 1.61] [NS]
Oral calcium (g)	0 [0 – 1]	0 [0 – 2] [NS]
IV calcium (ml)	20 [20 – 20]	20 [19 – 20] [NS]

Data are presented as mean (SD) or median [IQR]; n=15 in each group.

5.4 SYMPTOMS AND ADVERSE EVENTS

There were no SAEs. None of the subjects' FBC results fell outwith the pre-specified safety parameters (Chapter 2, Page 96).

The frequencies of reported symptoms did not vary significantly between the groups (Table 5C). All symptoms were mild, predictable from the procedures involved and of short duration. No subjects reported breathlessness or wheeze. One subject from the sham group developed a cough just over an hour after LPS inhalation which was associated with a fall in FEV_1 >10% below baseline; however, he had no audible wheeze on auscultation and both the cough and fall in FEV_1 resolved spontaneously within the next 2 hours.

Paracetamol was given to 5 subjects in the depletion group (including one subject who was given two separate doses), compared to 8 subjects in the sham group.

Table 5C: Symptoms and adverse events occurring in each group

SYMPTOM/ADVERSE EVENT	SHAM GROUP	DEPLETION GROUP
Pyrexial symptoms *	10 (67%)	5 (33%)
Paraesthesiae (digital/facial) †	8 (53%)	7 (47%)
Headache	8 (53%)	6 (40%)
Sore throat	7 (47%)	9 (60%)
Cough ‡	5 (33%)	6 (40%)
Light-headedness **	4 (27%)	4 (27%)
Chest tightness ††	4 (27%)	2 (13%)
Fall in FEV_1 >10% below baseline ‡‡	4 (27%)	0 (0%)
Desaturation <92% ***	3 (20%)	3 (20%)
Bruising/discomfort due to venepuncture	3 (20%)	2 (13%)
Nausea	3 (20%)	0 (0%)
Myalgia	1 (7%)	1 (7%)

All symptoms/AEs were mild and resolved fully; they were grouped (post-hoc) into categories and are expressed as frequency (%); n=15 each group; statistical analysis by Fisher's exact test demonstrated no significant differences between the groups in the frequency of any symptom/adverse event.

* 7 further subjects in the depletion group and the remaining 5 subjects in the sham group did not report pyrexial symptoms despite recording a temperature >37.4 °C during their study day.

† all episodes of paraesthesiae began during leukapheresis and resolved with treatment of presumed citrate-related hypocalcaemia.

‡ this does not include cough during bronchoscopy.

** one subject from the depletion group developed a vasovagal episode with sudden-onset light-headedness during leukapheresis, plus transient bradycardia and hypotension which resolved rapidly on lying flat. He was given 300ml 0.9% saline IV and, in the interests of safety, leukapheresis was not recommenced.

†† described as a sensation of slight restriction upon deep inspiration.

‡‡ associated with chest tightness in three subjects (and precluding bronchoscopy in one subject but occurring after bronchoscopy in the other two subjects).

*** during bronchoscopy; all episodes were transient (resolving within 2 minutes either spontaneously or with an increase in supplemental oxygen from 2 to 4 litres/minute); all subjects had SaO_2 ≥94% breathing room air by 2 hours post-procedure.

5.5 THE EFFECTS OF MNC DEPLETION ON CLINICAL PARAMETERS

LPS inhalation caused a sharp rise in mean HR in the sham group subjects, from 74 (SD 9) minute^{-1} at baseline to a peak mean HR of 100 (11) minute^{-1} (P<0.0001). A very similar pattern occurred in the depletion group subjects, where mean HR at baseline was 72 (SD 9) minute^{-1}, compared to a peak of 97 (9) minute^{-1} (P=0.52 for between-groups comparison) (Figure 5b, Panel A). HR had returned to baseline levels by 24 hours in both the sham and depletion groups (mean HR of 70 (SD 10) minute^{-1} and 67 (SD 11) minute^{-1} respectively).

As expected, LPS inhalation caused a clear rise in mean temperature in subjects from the sham group, from 36.2 (SD 0.5) °C at baseline to a mean peak temperature of 38.3 (0.5) °C (P<0.0001). MNC depletion did not affect this LPS-induced rise in temperature; the mean temperature of subjects in the depletion group was 36.4 (SD 0.5) °C at baseline with a peak of 38.0 (0.6) °C (P=0.11 for between-groups comparison) (Figure 5b, Panel B). By 24 hours, mean temperature had fallen to baseline levels in both groups (36.3 (SD 0.3) °C in the sham group and 36.5 (0.4) °C in the depletion group).

LPS inhalation caused a small rise in median RR in the sham group subjects, from a median of 14 [IQR 12-14] minute^{-1} at baseline to a peak of 20 [16–20] minute^{-1} (P<0.0001). A very similar pattern was seen in the depletion group subjects, with a baseline median RR of 14 [IQR 14-14] minute^{-1} and a peak median RR of 18 [16-20] minute^{-1} (P=0.79 for between-groups comparison) (Figure 5b, Panel C). Median RR at 24 hours was similar to baseline readings in all subjects (14 [IQR 14-16] minute^{-1} for both groups).

Mean baseline SaO_2 were 98 (SD 1) % in both groups. During and/or after bronchoscopy, there were small falls in mean SaO_2 readings in both groups (mean nadir of 95 (SD 2) % in the sham group and 96 (1) % in the depletion group (P=0.16 for between-groups comparison) (Figure 5b, Panel D). Mean SaO_2 at 24 hours had recovered to baseline levels in both groups.

Mean sBP at baseline was similar in both groups (130 (SD 10) mmHg in the sham group, compared to 134 (15) mmHg in the depletion group); the lowest recorded sBP was slightly lower in both groups (mean of 117 (SD 11) mmHg in the sham group and 114 (11) mmHg in the depletion group; between-groups comparison P=0.14) (Figure 5b, Panel E). In both groups, mean sBP had risen again by 24 hours (127 (SD 8) mmHg in the sham group and 124 (10) mmHg in the depletion group).

The maximal fall in mean FEV_1 was less than 10% in both groups, falling from a mean FEV_1 of 4.78 (SD 0.51) L at baseline to a nadir of 4.42 (0.57) L in the sham group, compared to a baseline mean FEV_1 of 4.72 (0.54) L and a nadir of 4.49 (0.57) L in the depletion group. There was a greater range in fall of FEV_1 in the sham group, but the overall between-groups comparison was not significant (P=0.08; Figure 5c). The maximal fall in mean FVC was also small, with a baseline mean FVC of 5.75 (SD 0.60) L and a nadir of 5.43 (0.60) L in the sham group, compared to a baseline mean FVC of 5.53 (0.64) L and a nadir of 5.38 (0.63) L in the depletion group. Again, there was a greater range in % fall in FVC in the sham group, which did not reach significance (P=0.06 for between-groups comparison; Figure 5c).

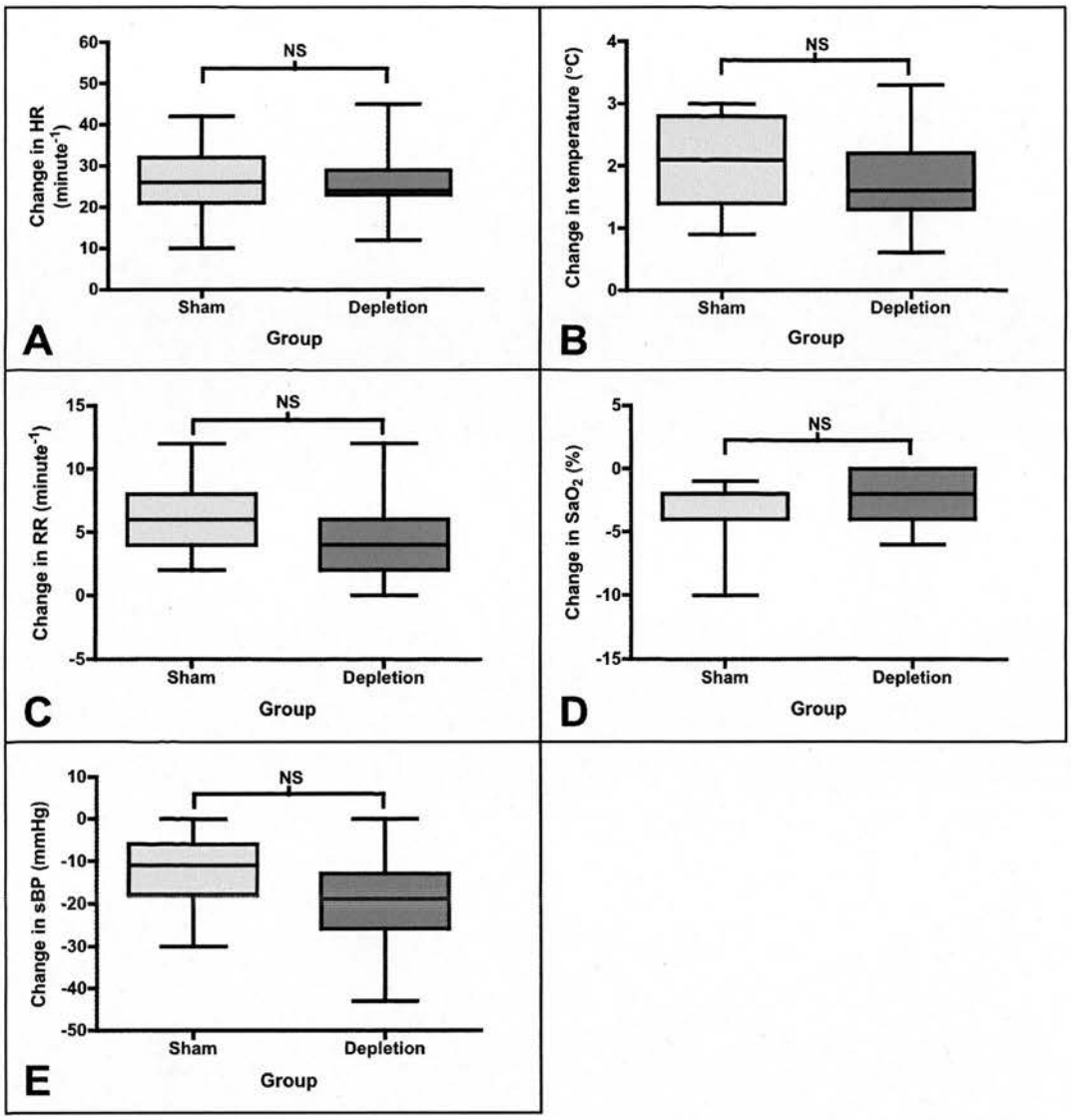


Figure 5b: Maximal changes in (A) HR, (B) temperature, (C) RR, (D) SaO_2 and (E) sBP during the study

Data are displayed as box (median/IQR) and whiskers (range); n=15 each group; between-groups statistical analysis was by ANCOVA, using baseline value as covariate.

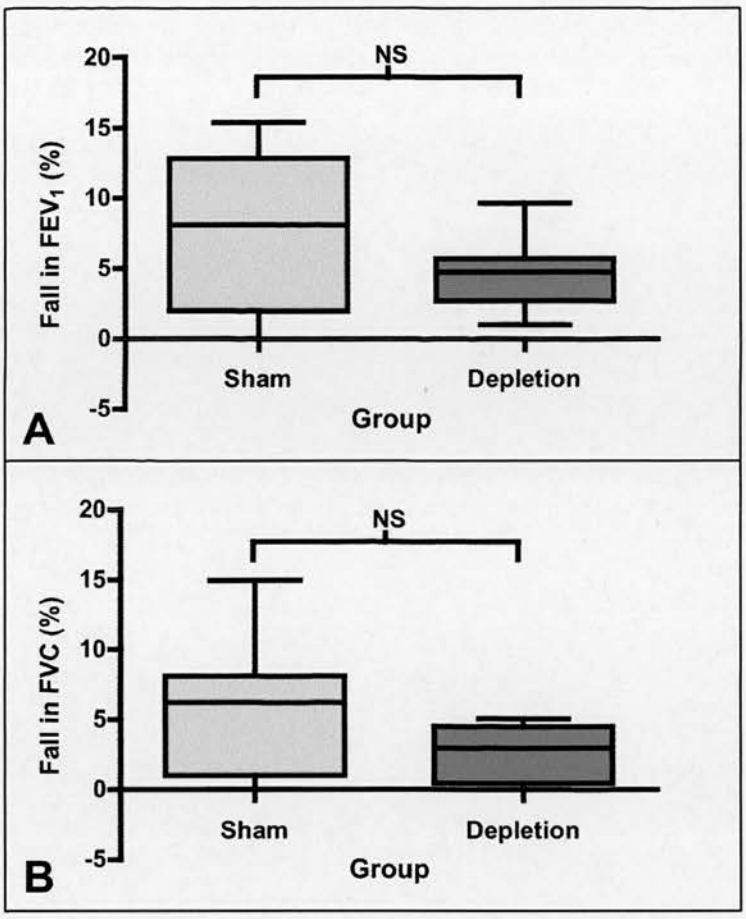


Figure 5c: Maximal % falls in (A) mean FEV_1 and (B) mean FVC during the study
Data are displayed as box (median/IQR) and whiskers (range); n=15 for both groups; between-groups statistical analysis was by ANCOVA, using baseline value as covariate.

5.6 CELL RETRIEVAL BY LEUKAPHERESIS

The volumes of the MNC collections obtained by leukapheresis in the 15 depletion group subjects were relatively consistent (median MNC collection volume of 0.319 [IQR 0.288 – 0.330] L) and similar to the volumes collected during Study B (see Page 147). The subject who had a vasovagal episode during leukapheresis was a notable outlier (2.3 TBVs were processed, resulting in an MNC collection volume of only 0.218 L).

The total cell numbers removed by leukapheresis (derived from the automated cell counts multiplied by the cell collection volumes) are presented in Table 5D. As expected from Study B (see Table 4C, Page 149), leukapheresis depleted large numbers of RBCs, platelets, lymphocytes and monocytes, but much smaller numbers of neutrophils, basophils and eosinophils.

Table 5D: Cell counts in the MNC collections of the depletion group subjects

CELL TYPE REMOVED	TOTAL CELL NUMBER ($x10^9$)
RBCs	187 [130 – 280]
Platelets	1013 (172)
Total WBCs	33.6 (10.6)
Lymphocytes	23.4 (7.27)
Monocytes	7.67 (2.33)
Neutrophils	0.74 [0 – 2.65]
Basophils	0.23 [0.17 – 0.28]
Eosinophils	0.003 [0.003 – 0.01]

Data are presented as median [IQR] or mean (SD); n=15 (depletion group only).

5.7 CELL YIELD FROM LEUKAPHERESIS

a) Monocyte yield

There was a strong correlation between the number of monocytes present in the MNC collections (i.e. depleted by leukapheresis) and the respective number of circulating monocytes in baseline blood (r=0.84 [$CI_{0.95}$ 0.57, 0.94]) (Figure 5d, Panel A). Monocyte yield was 2.3, i.e. the mean absolute number of monocytes depleted by leukapheresis (7.67 (SD 2.33) $x10^9$) was equivalent to 2.3 times the

mean absolute number of monocytes circulating in blood at baseline (3.32 (0.92) $x10^9$).

b) Lymphocyte yield

There was also a strong correlation between the number of lymphocytes present in the MNC collections and the respective number in blood at baseline (r=0.83 [$CI_{0.95}$ 0.55, 0.94) (Figure 5d, Panel B). Lymphocyte yield was 1.8 i.e. the mean absolute number of lymphocytes removed by leukapheresis (23.4 (SD 7.3) $x10^9$) was equivalent to 1.8 times the mean absolute number of lymphocytes circulating in baseline blood (13.1 (2.8) $x10^9$).

c) Platelet yield

The number of platelets present in the MNC collections also correlated with the respective number in blood at baseline (r=0.65 [$CI_{0.95}$ 0.21, 0.87]) (Figure 5d, Panel C). Platelet yield was 0.8 (the mean absolute number of platelets removed by leukapheresis (1013 (SD 172) $x10^9$) was 0.8 times the mean absolute number of platelets circulating in baseline blood (1244 (222) $x10^9$).

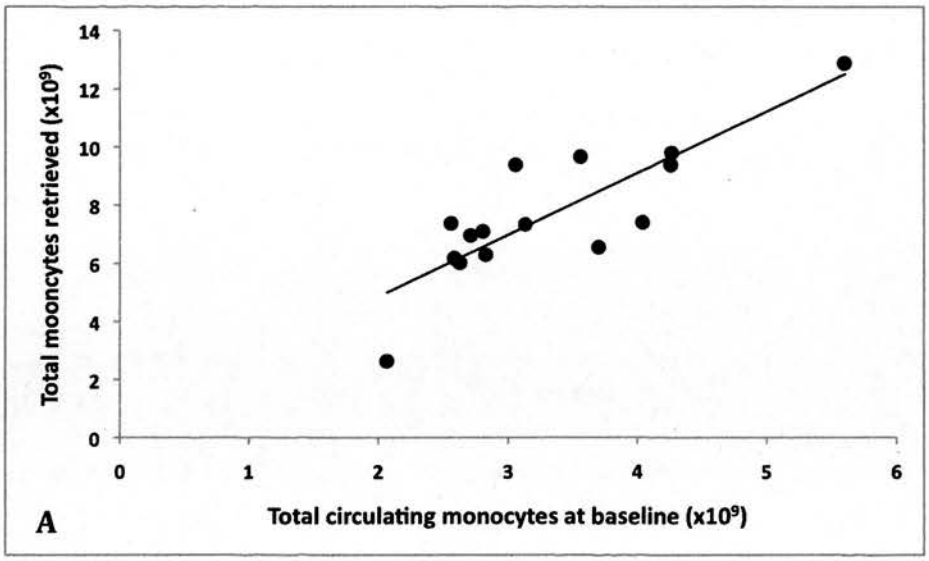
d) Neutrophil yield

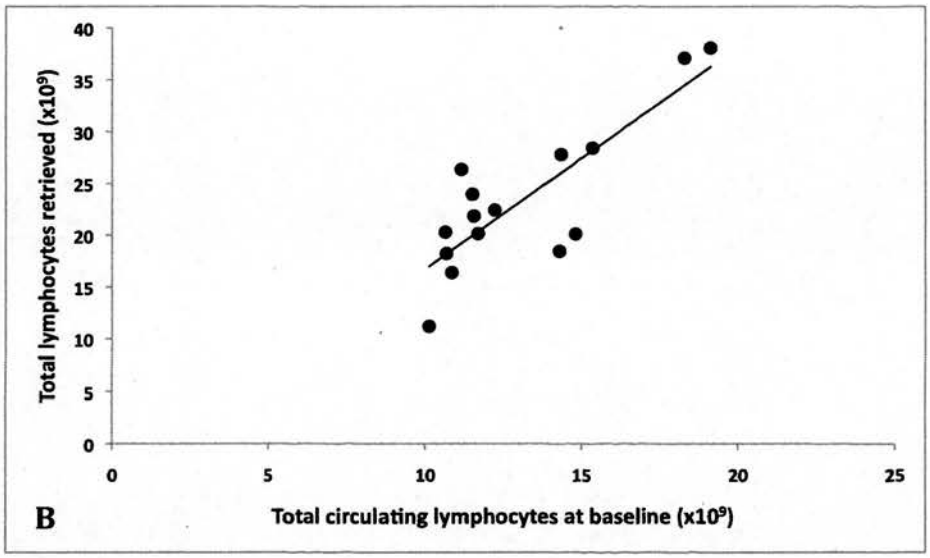
There was only a very weak correlation between the number of neutrophils present in the MNC collections and the respective number in baseline blood (r=0.3 [$CI_{0.95}$ -0.27, 0.69]) (Figure 5d, Panel D). Indeed, six of the fifteen MNC collections were completely free of neutrophils. The median absolute number of

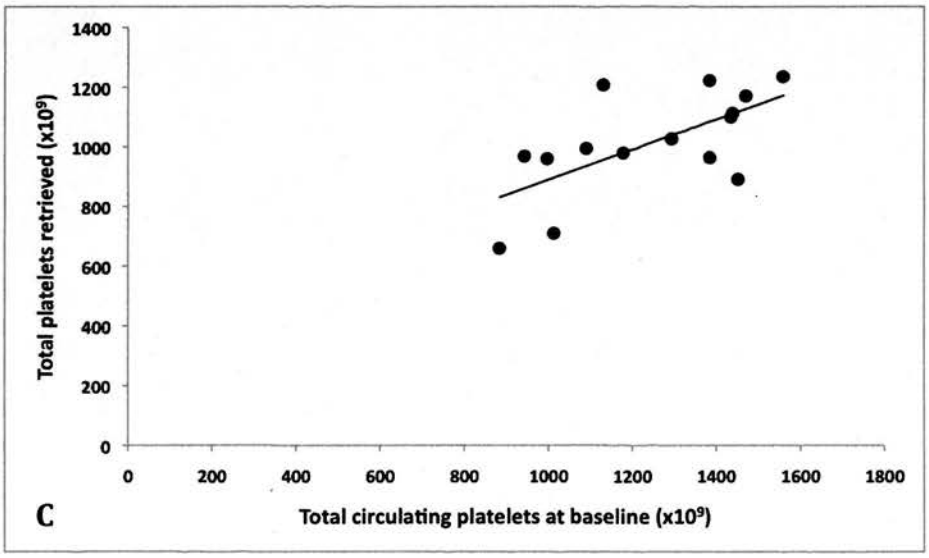
neutrophils removed by leukapheresis (0.74 [IQR 0 – 2.65] $x10^9$) was only 0.05 times the median absolute number of neutrophils circulating in baseline blood (16.2 [12.8 – 21.7] $x10^9$).

e) RBC yield

The number of RBCs retrieved by leukapheresis also showed only a very weak correlation with the respective number in blood at baseline (r=0.2 [$CI_{0.95}$ -0.36, 0.64]) (Figure 5d, Panel E). Indeed, the median absolute number of RBCs present in the MNC collections (0.19 [0.13 – 0.28] $x10^{12}$) was just 0.007 times the median absolute number of RBCs circulating in baseline blood (26.9 [25.9 – 29.1] $x10^{12}$).


Total mooncytes retrieved (x10⁹)
14
12
10
8
6
4
2
0
0
1
2
3
4
5
6
A
Total circulating monocytes at baseline (x10⁹)


Total lymphocytes retrieved (x10⁹)
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
B
Total circulating lymphocytes at baseline (x10⁹)


Total platelets retrieved (x10⁹)
1400
1200
1000
800
600
400
200
0
0
200
400
600
800
1000
1200
1400
1600
1800
C
Total circulating platelets at baseline (x10⁹)

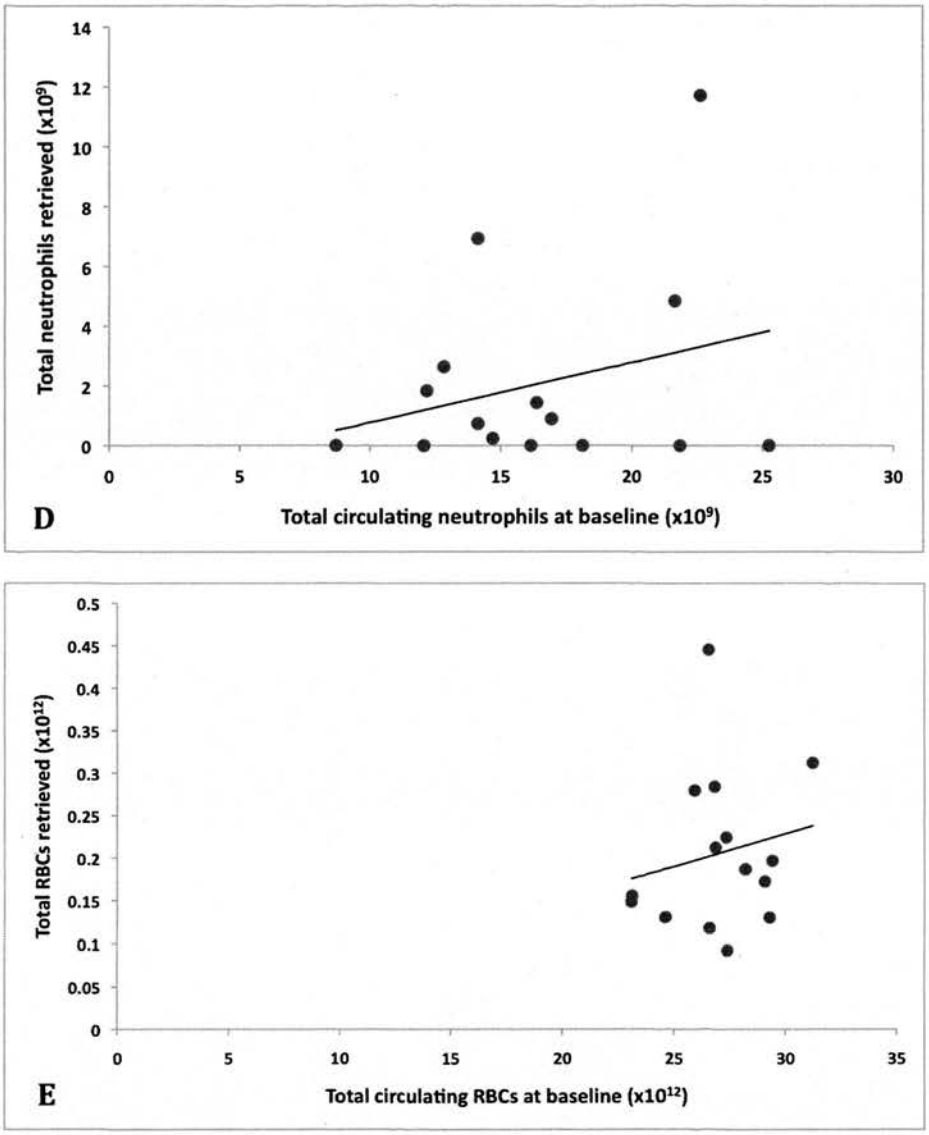


Figure 5d: Cell counts within the MNC collections compared with circulating counts in baseline blood, for (A) monocytes, (B) lymphocytes, (C) platelets, (D) neutrophils and (E) RBCs

n=15 (depletion group only); statistical analysis of correlation was by Pearson's test.

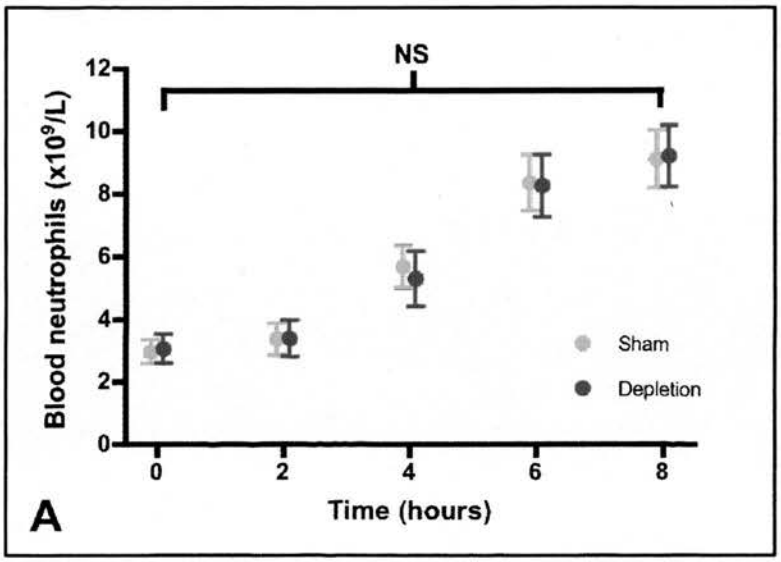
5.8 THE EFFECTS OF MNC DEPLETION AS MEASURED IN BLOOD

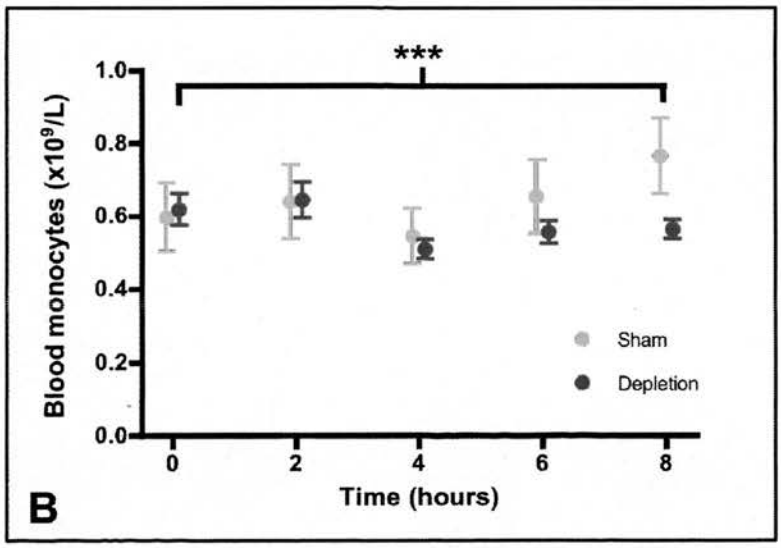
a) Circulating blood neutrophil counts

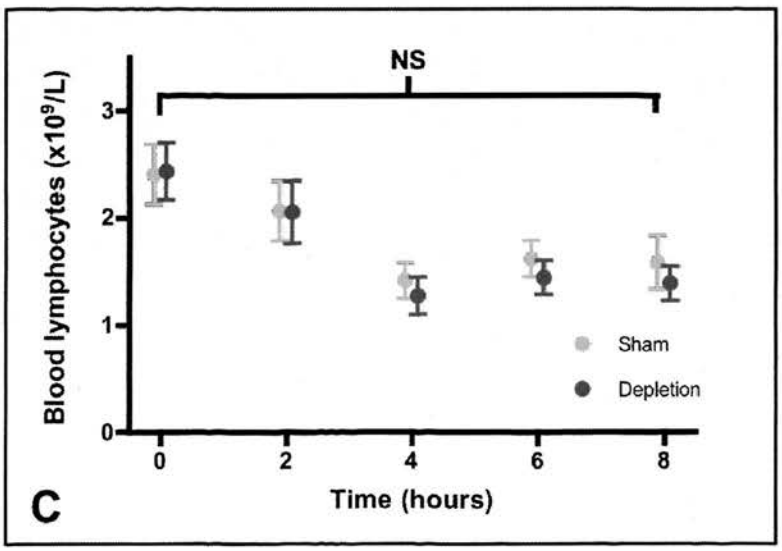
As expected, mean blood neutrophil count rose steeply after LPS inhalation, from 2.97 (SD 0.68) $x10^9$/L at baseline to 9.12 (1.65) $x10^9$/L at 8 hours in the sham group subjects ($P<0.0001$). Leukapheresis did not alter the primary end-point: the mean circulating blood neutrophil count at baseline was 3.06 (SD 0.84) $x10^9$/L compared to 9.22 (1.76) $x10^9$/L at 8 hours in the depletion group ($P=1.00$ for the between-groups comparison) (Figure 5e, Panel A).

Since neutrophils, albeit in small quantities, were sequestered in the MNC collections of the subjects from the depletion group, this may have altered the 8-hour circulating blood neutrophil counts and thus affected the primary end-point. A second analysis was therefore performed to correct for the estimated numbers of neutrophils directly removed by leukapheresis, which were added to the respective 8-hour blood neutrophil counts to calculate adjusted blood neutrophil counts at 8 hours (mean of 9.59 (SD 1.96) $x10^9$/L). Even after this correction for neutrophil removal, MNC depletion continued to have no effect on the LPS-induced rise in blood neutrophil count ($P=0.55$ for between-groups analysis).

A repeated-measures analysis, incorporating circulating blood neutrophil counts at all the intermediate (2, 4 and 6-hour) time-points, confirmed that MNC depletion had no effect on the LPS-induced rise in blood neutrophil count over the entire 8-hour period when compared to sham ($P=0.67$).


NS
Blood neutrophils (x10⁹/L)
12
10
8
6
4
2
0
0
2
4
6
8
Time (hours)
Sham
Depletion
A


***
Blood monocytes (x10⁹/L)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
Time (hours)
Sham
Depletion
B


NS
Blood lymphocytes (x10⁹/L)
3
2
1
0
0
2
4
6
8
Time (hours)
Sham
Depletion
C

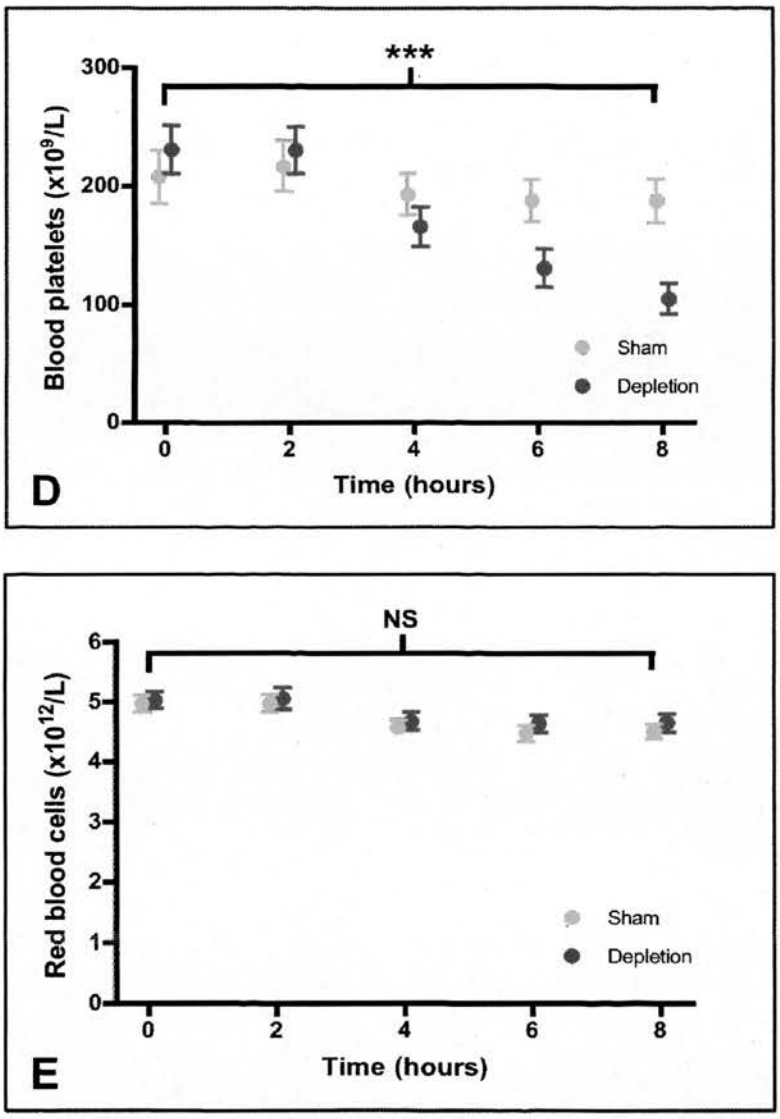


Figure 5e: Circulating blood (A) neutrophil, (B) monocyte, (C) lymphocyte, (D) platelet and (E) RBC counts between baseline and 8 hours

Circles and error bars represent means and $CI_{0.95}$; n=15 for each group at each time-point, except sham at 2 hours where n=14 (one blood sample clotted prior to processing); between-groups statistical analysis was by ANCOVA, comparing the change in circulating blood cell count between baseline and 8 hours, using baseline count as covariate; *** $P<0.0005$.

By 24 hours, circulating blood neutrophil counts in the sham group subjects had begun to fall, although mean blood neutrophil count remained significantly elevated above baseline level (7.73 (SD 1.9) $x10^9/L$), possibly due to the effects of intervening BAL. In the depletion group, mean circulating blood neutrophil

count at 24 hours was slightly lower, at 6.47 (SD 2.3) $x10^9$/L, although this did not reach significance (P=0.09 for between-groups comparison with baseline).

b) Circulating blood monocyte counts

Mean blood monocyte count in the sham group subjects rose from 0.60 (SD 0.17) $x10^9$/L at baseline to 0.77 (0.19) $x10^9$/L at 8 hours (P<0.005). Conversely, in the depletion group subjects (as would be expected by the large numbers of monocytes removed during leukapheresis), mean blood monocyte count fell from 0.62 (SD 0.17) $x10^9$/L at baseline to 0.56 (0.10) $x10^9$/L at 8 hours (P=0.0001 for between-groups comparison) (Figure 5e, Panel B).

By 24 hours, mean circulating blood monocyte count remained elevated in the sham group subjects (0.78 (SD 0.24) $x10^9$/L) and, despite the earlier fall at 8 hours, was also elevated above baseline levels in subjects from the depletion group (0.68 (0.23) $x10^9$/L; between-groups comparison with baseline P=0.73).

c) Circulating blood lymphocyte counts

Mean blood lymphocyte count fell after LPS inhalation in the sham group subjects, from a baseline of 2.41 (SD 0.50) $x10^9$/L to 1.59 (0.46) $x10^9$/L by 8 hours (P<0.0001). Mean blood lymphocyte count also fell in the depletion group subjects, but not to a significantly greater extent (baseline mean of 2.44 (SD 0.48) $x10^9$/L, compared to 1.39 (0.29) $x10^9$/L at 8 hours; P=0.10 for the between-groups comparison) (Figure 5e, Panel C). By 24 hours, mean circulating blood lymphocyte count in the sham group subjects had partially

recovered, rising to 2.08 (SD 0.43) $x10^9$/L. In the depletion group subjects, however, mean blood lymphocyte count had risen to a much lesser extent by 24 hours, remaining significantly below baseline levels (1.77 (SD 0.38) $x10^9$/L; $P<0.01$ for between-groups comparison with baseline).

d) Circulating blood platelet counts

After LPS inhalation, mean blood platelet count in the sham group was similar at baseline compared to 8 hours (208 (SD 40) $x10^9$/L and 187 (33) $x10^9$/L respectively; $P=0.11$). In comparison, there was a dramatic fall in mean blood platelet count by 8 hours in the depletion group subjects, from 231 (SD 37) $x10^9$/L at baseline to 105 (23) $x10^9$/L ($P<0.0001$ for the between-arms comparison) (Figure 5e, Panel D). By 24 hours, mean circulating blood platelet count again remained relatively stable in the sham group subjects, at 193 (SD 35) $x10^9$/L. In the depletion group subjects, mean blood platelet count remained much lower than baseline at 24 hours (115 (SD 24) $x10^9$/L; $P<0.0001$ for the between-groups comparison with baseline).

e) Circulating RBC counts

There was a significant fall in mean RBC count by 8 hours in the sham group, from 4.97 (SD 0.25) $x10^{12}$/L at baseline to 4.50 (0.22) $x10^{12}$/L ($P<0.0001$). Mean RBC count fell to a similar extent in the depletion group, to 4.65 (SD 0.27) $x10^{12}$/L at 8 hours, from a baseline of 5.03 (0.25) $x10^{12}$/L ($P=0.12$ for the between-groups comparison) (Figure 5e, Panel E). By 24 hours, in both groups, mean circulating RBC count had risen towards baseline levels (4.76 (SD 0.28)

$x10^{12}$/L in the sham group and 4.92 (0.32) $x10^{12}$/L in the depletion group; *P*=0.14 for between-groups comparison with baseline).

f) Blood monocyte subsets

The proportions of blood monocyte subsets, as a proportion of total HLA-DR$^+$ monocytes, were determined at each time-point using flow cytometry. The baseline proportion of classical monocytes was 91.4 (SD 1.7) % in the sham group and 90.2 (3.6) % in the depletion group, similar to the baseline proportions observed in the Study A and Study B subjects. There were only very small changes in the proportions of each monocyte subset in blood over time, with no clear effect from MNC depletion.

g) Plasma C-reactive protein levels

There was a steep rise in plasma CRP levels by 24 hours after LPS inhalation in subjects from the sham group (34.8 (SD 15.3) ng/ml, versus 1.1 (1.3) ng/ml at baseline; n=14, *P*<0.0001). This rise in plasma CRP was not influenced by MNC depletion, with CRP levels increasing to a similar extent, from 0.9 (SD 0.8) ng/ml at baseline to 34.6 (17.1) ng/ml at 24 hours in the depletion group subjects (n=14; *P*=0.90).

h) Inflammatory and cell injury markers in plasma

The levels of several pro-/anti-inflammatory and chemotactic cytokines, soluble cell adhesion molecules, markers of neutrophil activation and markers of

epithelial and endothelial cell injury were measured in plasma at baseline and 8 hours (Table 5E). Levels of the major cytokines were also measured at 24 hours.

Plasma levels of the pro-inflammatory cytokines TNFα and IL-1β and the anti-inflammatory cytokines IL-12p70 and IL-10 were undetectable at baseline, did not reach detectable levels at 8 hours and remained undetectable at 24 hours. There were, however, small but clear rises in the plasma levels of the pro-inflammatory cytokines IL-6 and IL-8 in both the sham group and depletion group subjects by 8 hours; levels of both had fallen again by 24 hours, and although levels of plasma IL-6 remained slightly higher than baseline in the sham group subjects, the pattern of change in plasma [IL-6] levels between 0 and 24 hours was not significantly different between the groups.

There was a non-significant trend towards lower plasma levels of MCP-1 (a chemotactic cytokine for monocytes) by 8 hours in both groups. Plasma levels of MIP-1α, another chemotactic cytokine, were undetectable in both groups at baseline and at 8 hours. In comparison, plasma levels of SDF-1α (a chemotactic cytokine for monocytes and lymphocytes) fell significantly between baseline and 8 hours in both groups.

There were significant rises in the levels of plasma HNE and MPO (markers of neutrophil activation and function) by 8 hours, which were comparable between the sham and depletion groups.

LPS inhalation had no effect, in either group, on plasma levels of the soluble cell adhesion molecule sE-selectin. Plasma sL-selectin levels, however, had fallen

significantly in both groups by 8 hours; there was a trend towards a greater fall in plasma sL-selectin levels in subjects from the sham group, although this did not reach statistical significance.

There was no significant difference in the 8-hour levels of plasma CC-16 (a marker of alveolar epithelial injury) in the sham and depletion groups. Levels of plasma RAGE (an injury marker for type I alveolar epithelial cells) rose significantly after LPS inhalation, to a similar extent in both groups; by 24 hours, plasma RAGE levels had fallen back towards baseline levels and, although this fall was not quite so marked in subjects from the sham group, there was no statistical difference between the groups. In contrast, LPS inhalation had no effect upon plasma levels of SP-D (a marker of alveolar epithelial type 2 cell injury) in subjects from either group. There was also no significant change in either group in the plasma levels of vWF (a marker of endothelial cell injury) after LPS inhalation.

Table 5E: Plasma concentrations of cytokines, cell adhesion molecules and markers of neutrophil activation and cell injury

MARKER	GROUP	PLASMA			BETWEEN-GROUPS ANALYSIS	
		Baseline	8 hours	24 hours	8 hours	24 hours
IL-1β (pg/ml)	Sham	Undetectable	Undetectable	Undetectable	N/A	N/A
	Depletion	Undetectable	Undetectable	Undetectable		
IL-6 (pg/ml)	Sham	Undetectable	6.5 [4.2 - 10.3] ***	2.8 [*x* – 3.8] *	*P*=0.86 †	*P*=0.39 †
	Depletion	Undetectable	7.7 [3.9 - 10.8] ***	*x* [*x* – 3.2] NS		
IL-8 (pg/ml)	Sham	*x* [*x* - 4.0]	5.2 [4.7 - 7.6] **	Undetectable	*P*=0.71 †	N/A
	Depletion	Undetectable	5.8 [*x* - 8.4] *	Undetectable		
TNFα (pg/ml)	Sham	Undetectable	Undetectable	Undetectable	N/A	N/A
	Depletion	Undetectable	Undetectable	Undetectable		
IL-10 (pg/ml)	Sham	Undetectable	Undetectable	Undetectable	N/A	N/A
	Depletion	Undetectable	Undetectable	Undetectable		
IL-12p70 (pg/ml)	Sham	Undetectable	Undetectable	Undetectable	N/A	N/A
	Depletion	Undetectable	Undetectable	Undetectable		
MCP-1 (pg/ml)	Sham	146 [129 - 215]	200 [165 - 247] NS	No assay	*P*=0.45	N/A
	Depletion	140 [118 - 152]	202 [146 - 259] NS			
MIP-1α (pg/ml)	Sham	Undetectable	Undetectable	No assay	N/A	N/A
	Depletion	Undetectable	Undetectable			
SDF-1α (pg/ml)	Sham	1910 [1810 - 2060]	1630 [1390 - 1790] ***	No assay	*P*=0.35	N/A
	Depletion	1700 [1530 - 1830]	1400 [1340 - 1600] *			

Table 5E continued...

MARKER	GROUP	PLASMA			BETWEEN-GROUPS ANALYSIS	
		Baseline	8 hours	24 hours	8 hours	24 hours
HNE (ng/ml)	Sham	174 [151 – 204]	265 [216 – 369] **	No assay	*P*=0.11	N/A
	Depletion	162 [129 – 204]	197 [157 – 302] *			
MPO (ng/ml)	Sham	23.5 [19.5 – 37.0]	165 [104 – 218] ***	No assay	*P* =0.83	N/A
	Depletion	20.0 [15.0 – 25.0]	106 [87.0 – 149] ***			
sE-selectin (ng/ml)	Sham	35.6 [28.3 – 48.3]	31.1 [23.3 – 44.6] NS	No assay	*P*=0.56	N/A
	Depletion	37.5 [28.5 – 53.2]	33.5 [26.5 – 45.3] NS			
sL-selectin (ng/ml)	Sham	894 [843 – 1110]	715 [696 – 881] ***	No assay	*P* =0.06	N/A
	Depletion	903 [846 – 1020]	776 [729 - 882] **			
CC-16 (ng/ml)	Sham	No assay	39.8 [34.1 – 64.3]	No assay	*P* =0.91 †	N/A
	Depletion		43.8 [35.6 – 55.4]			
RAGE (pg/ml)	Sham	1320 [773 – 1870]	2090 [1400 – 1770] ***	1630 [1150 – 2070] *	*P* =0.48	*P* =0.64
	Depletion	1510 [913 – 2210]	2030 [1460 – 2930] **	1590 [1060 – 2330] NS		
SP-D (ng/ml)	Sham	448 [245 – 856]	425 [214 – 749] NS	No assay	*P* =0.98	N/A
	Depletion	468 [286 – 632]	450 [267 – 890] NS			
vWF (mU/ml)	Sham	907 [634 – 1350]	910 [694 – 1400] NS	No assay	*P* =0.18	N/A
	Depletion	832 [454 – 1390]	741 [527 – 2070] NS			

Data are expressed as median [IQR]; *x* denotes a reading below the lower limit of detection of the assay; n=14 at individual time-points and n=13 for paired analyses between time-points for the sham group; n≥14 for the depletion group. Within-group statistical analyses (between 0 and 8 hours or between 0 and 24 hours) are by Wilcoxon signed-rank test; between-group statistical analyses were by ANCOVA using baseline value as covariate, unless baseline levels were undetectable or not performed († MWU test performed instead on data from a single time-point); *P<0.05, **P<0.005, ***P<0.0005.

5.9 THE EFFECTS OF MNC DEPLETION AS MEASURED IN BAL FLUID

a) BAL volume and total cell count

Similar volumes of BAL fluid were retrieved from the subjects in the two groups during bronchoscopy (mean BAL volume of 86 (SD 25) ml in the sham group, compared to 83 (37) ml in the depletion group; *P*=0.78) (Figure 5f). These volumes were similar to those collected in Studies A and B (see Chapter 3, Page 124 and Chapter 4, Page 158).

Cell viability was identical in both groups (mean of 92 (SD 4) %). MNC depletion did not significantly reduce the total viable cell count in BAL fluid, which was 4.5 (SD 2.1) $x10^5$/ml in the sham group and 3.9 (1.3) $x10^5$/ml in the depletion group; *P*=0.39 (Figure 5f). Total viable cell counts were similar, possibly even slightly higher, than those seen in the LPS group from Study A (see Chapter 3, Page 124).

b) Levels of LPS detectable in BAL fluid supernatant

A semi-quantitative LAL test was used to determine the levels of LPS detectable in BAL fluid supernatant from the subjects in each study group. LPS was detectable in all the BAL fluid supernatant samples and at an equivalent concentration in all subjects (≥0.25 but <0.50 endotoxin units/ml).

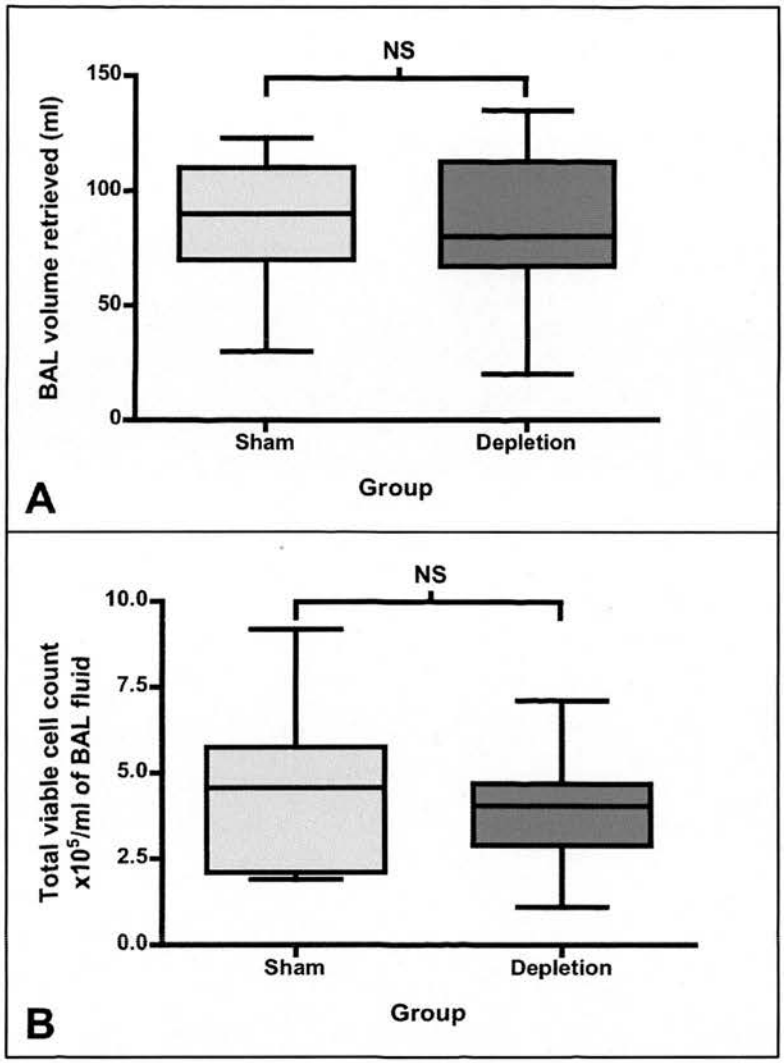


Figure 5f: BAL volumes retrieved (Panel A) and total viable cell counts per ml of BAL fluid (Panel B)

Data are presented as box (median/IQR) and whiskers (range); n=14 for the sham group (one subject did not undergo bronchoscopy due to a >10% fall in FEV_1 pre-procedure); n=15 for the depletion group; statistical analysis was by 2-sample t-test.

c) BAL cell content (light microscopy)

BAL cell content was calculated by examination of cytospins using light microscopy to obtain differential cell counts. The proportions of each cell type are shown in Table 5F. Alveolar macrophages represented the majority of BAL cells in both the sham and depletion groups. As expected after LPS inhalation, there was also a relatively high neutrophil content in BAL from the sham group.

BAL neutrophil content was very similar in the depletion group and MNC depletion therefore did not appear to reduce LPS-induced BAL neutrophilia (a major secondary end-point of the study). The proportion of mononuclear cells present in BAL was slightly lower in the depletion group than the sham group.

Table 5F: Relative proportions of each cell type present in BAL fluid

CELL TYPE	SHAM GROUP	DEPLETION GROUP
Alveolar macrophages	62 (19)	66 (17) ^{NS}
Mononuclear cells	4.8 (2.2)	3.5 (1.6) *
Neutrophils	33 (18)	31 (17) ^{NS}

Data are expressed as mean (SD) % of total BAL cells; n=14 (sham group) and n=15 (depletion group); statistical analysis was by 2-sample t-test; * $P<0.05$.

Estimated absolute numbers of each cell type (per ml of BAL fluid) were obtained by adjusting the cell proportion data for total cell count (Figure 5g). The estimated numbers of AMs remained similar between groups, with a median of 231 [IQR 180 – 302] x10^3/ml in the sham arm and 238 [190 – 292] in the depletion arm (P=0.95). Adjusting for total cell count revealed wide variations in the estimated numbers of neutrophils in BAL fluid, with a median of 160 [IQR 33.6 – 240] x10^3/ml in the sham arm compared to 120 [64.5 – 152] x10^3/ml in the depletion arm (P=0.47). In line with the cell proportion data, the estimated number of mononuclear cells in BAL fluid was also smaller in the depletion group (median 13.5 [IQR 7.4 – 18.8] x10^3/ml) compared to the sham group (25.0 [7.6 – 32.8] x10^3/ml; P=0.02).

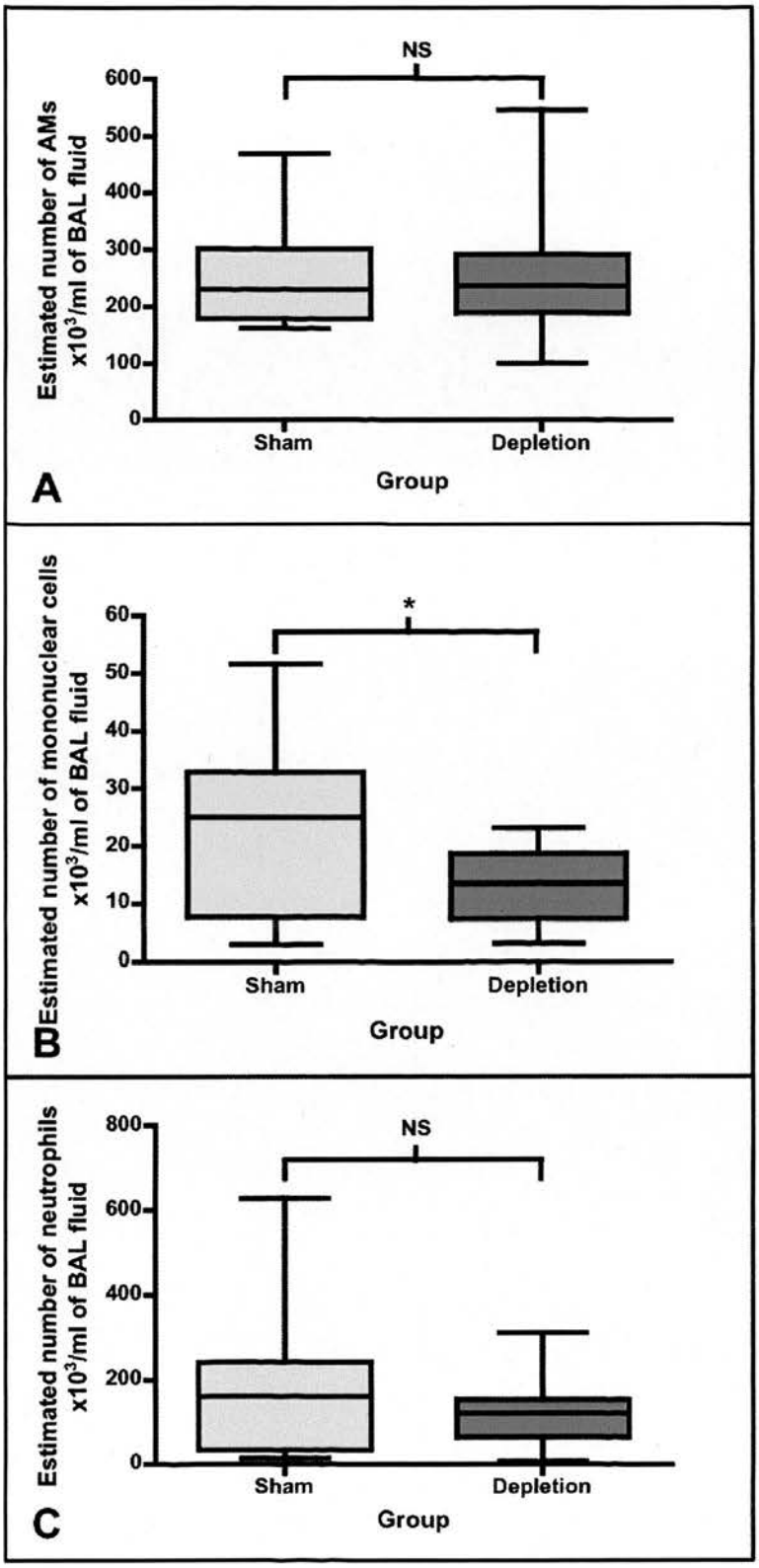


Figure 5g: Estimated absolute numbers of (A) AMs, (B) MNCs and (C) neutrophils in BAL fluid

Data are presented as box (median/IQR) and whiskers (range) plots; n=14 from the sham group and n=15 from the depletion group; statistical analysis was by 2-sample t-test; * $P<0.05$.

d) BAL cell content (flow cytometry)

As demonstrated in the preliminary studies, flow cytometry enabled accurate distinction of PMLCs from AMs and lymphocytes by plotting cell size and granularity. As before, relative cell surface antigen expression of CD14 and CD16 was then used to distinguish the two PMLC subtypes.

In the sham group subjects, the FSC-A versus SSC-A flow cytometry plots demonstrated prominent populations of neutrophils and PMLCs similar to that seen after LPS inhalation in Study A (an example plot is shown in Figure 5h); a visually identical pattern was seen in the depletion subjects.

As before, the HLA-DR$^+$ PMLCs could be easily divided by relative CD14 and CD16 expression into rPMLCs (CD14^{++}CD16$^+$, similar to intermediate blood monocytes) and iPMLCs (CD14^{++}CD16$^-$, similar to classical blood monocytes) (Figure 5h). In both the sham and depletion groups, iPMLCs made up the major PMLC subtype, with relatively few rPMLCs (Table 5G). The relative proportions of PMLC subtypes were very similar to those in the LPS group from Study A (see Table 3F, Page 131) and, as in the preliminary study, there was no clear PMLC population equivalent to CD14$^+$CD16^{++} (non-classical) blood monocytes.

Table 5G: Relative proportions of each PMLC subtype in BAL fluid

PMLC SUBTYPE	SHAM GROUP	DEPLETION GROUP
rPMLC	8.8 (3.9)	8.4 (4.6) ^{NS}
iPMLC	91.2 (3.9)	91.6 (4.6) ^{NS}

Data are expressed as mean (SD) % of total PMLCs in BAL; n=14 (sham group) and n=13 (depletion group); statistical analysis was by 2-sample t-test.

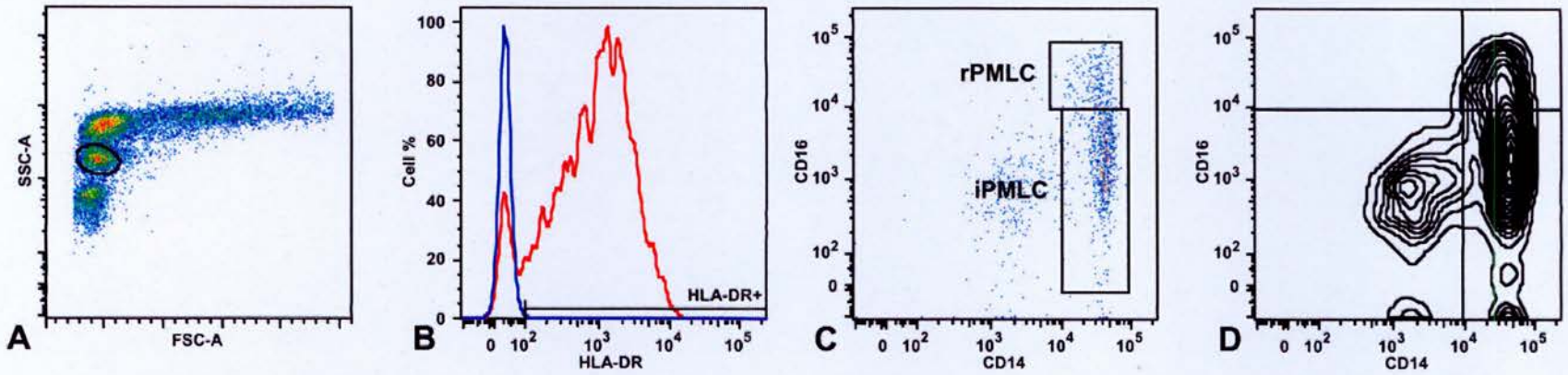


Figure 5h: Pulmonary monocyte-like cells (PMLCs) and their subtypes, identified by flow cytometry

The panels are an example of a set of flow plots for one of the sham group subjects. Panel A demonstrates the PMLC population on a simple FSC-A versus SSC-A plot (with lymphocytes lying inferiorly, neutrophils lying directly superior and AMs positioned superiorly to the right). Any HLA-DR$^-$ cells, including neutrophils, were then excluded from analysis (Panel B). The two PMLC subtypes were then distinguishable by their relative CD14 and CD16 expression (Panel C and, in the form of a contour plot, Panel D). The iPMLC subtype was equally predominant in the BAL fluid of subjects from the sham and depletion groups.

There was no significant difference in the mean proportion of T_{reg} cells in BAL from sham group subjects (4.8 (SD 2.8) %) compared to depletion group subjects (4.8 (2.1) %; *P*=0.97) and these proportions were similar to that found in the LPS group from Study A (see Chapter 3, Page 132).

e) Levels of protein in BAL fluid supernatant

The mean concentration of BAL protein in the sham group subjects was 0.24 (SD 0.13) g/L, a similar level to that seen after LPS inhalation in Study A. MNC depletion had no significant effect on mean BAL protein content (0.20 (SD 0.05) g/L; *P*=0.30) (Figure 5i). Urea levels in plasma and BAL supernatant were used to calculate the volume of ELF within each BAL sample (see Methods, Chapter 2, Page 103). Even after adjusting for BAL dilution, the mean ELF protein content did not differ significantly between groups (median of 2.40 [1.34 – 3.46] g/L in the sham group and 2.31 [1.30 – 3.29] g/L in the depletion group; *P*=0.82).

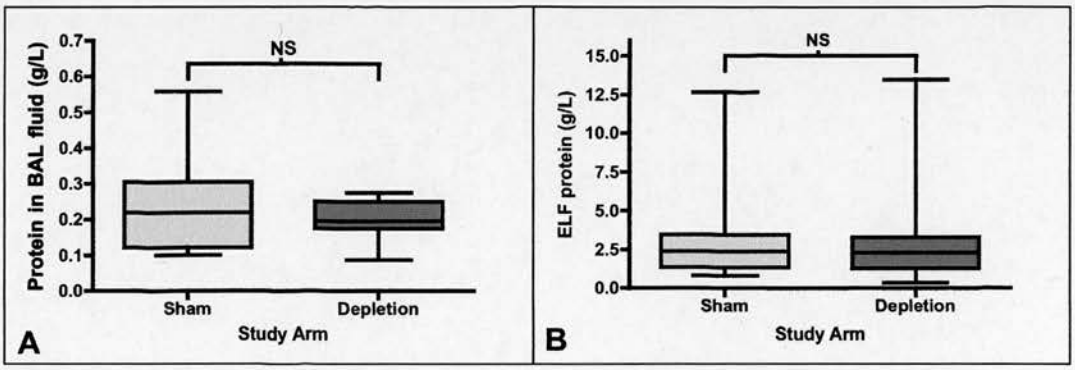


Figure 5i: Protein content within (A) BAL fluid supernatant and (B) within ELF
Data are presented as box (median/IQR) and whisker (range) plots; n=13 or n=14 (sham group, BAL and ELF results respectively) and n=15 (depletion group, all data); statistical analysis was by 2-sample t-test.

f) Markers of inflammation and cellular injury in BAL fluid supernatant

The levels of several pro-/anti-inflammatory and chemotactic cytokines, soluble cell adhesion molecules, markers of neutrophil activation and markers of epithelial and endothelial cell injury were measured in BAL fluid supernatant (Table 5H).

Table 5H: BAL fluid supernatant concentrations of cytokines, cell adhesion molecules and markers of neutrophil activation and cell injury

CYTOKINE/MARKER	SHAM GROUP	DEPLETION GROUP
IL-1β (pg/ml)	14.5 [8.30 – 26.7]	13.3 [10.2 – 15.4]
IL-6 (pg/ml)	338 [149 – 538]	293 [228 – 738]
IL-8 (pg/ml)	60.5 [50.0 – 180]	123 [100 – 133]
TNFα (pg/ml)	3.80 [*x* – 4.75]	*x* [*x* – 4.90]
IL-10 (pg/ml)	Undetectable	Undetectable
IL-12p70 (pg/ml)	2.65 [2.30 – 3.10]	2.70 [*x* – 3.0]
MCP-1 (pg/ml)	285 [156 – 502]	283 [183 – 329]
MIP-1α (pg/ml)	93.0 [22.5 – 110]	78.0 [49.0 – 175]
SDF-1α (pg/ml)	*x* [*x* – 48.0]	*x* [*x* – 29.1]
HNE (ng/ml)	150 [83.5 – 248]	112 [56.0 – 149]
MPO (ng/ml)	381 [187 – 733]	300 [192 – 441]
sE-selectin (ng/ml)	Undetectable	Undetectable
sL-selectin (ng/ml)	10.3 [4.05 – 16.1]	7.70 [5.30 – 10.0]
CC-16 (ng/ml)	1280 [810 – 1970]	1400 [825 – 2120]
RAGE (pg/ml)	2910 [1970 – 4290]	3360 [1800 – 6000]
SP-D (ng/ml)	3420 [2540 – 5990]	4140 [3130 – 5870]
vWF (mU/ml)	3.40 [*x* – 7.24]	*x* [*x* – 4.17]

Data are expressed as median [IQR] concentrations; n=14 (sham group) and n=15 (depletion group); *x* denotes a value below the lower limit of detection of the assay; between-groups statistical analysis was by MWU test and demonstrated no significance for any of the cytokines/markers (even after adjustment for BAL dilution).

5.10 THE EFFECT OF MNC DEPLETION AS MEASURED BY ^{18}F[FDG] PET

PET-CT scans were performed on 20 subjects (n=10 each group). Baseline characteristics for each sub-group remained well matched between the sham and depletion arms and were representative of overall parent group. The mean amount of ^{18}F[FDG] administered was consistent between the groups (190 (SD 17.3) MBq in the sham arm, compared to 188 (16.9) MBq in the depletion arm). In both groups, there was a mean time of 27 (SD 1) hours between baseline and commencing the PET-CT scan.

As discussed in Chapter 2 (Page 91), several methods were used to estimate pulmonary uptake of [^{18}F]FDG: Patlak analysis-derived influx constant (K_i) and intercept-adjusted K_i (to account for the initial volume of [^{18}F]FDG distribution) (Figure 5j) and also SUV and tissue-to-plasma activity ratio (TPR). With each method, estimates of [^{18}F]FDG uptake were performed for whole lung (incorporating the regions of interest from both lungs) and for left lung alone (to exclude any effect from prior BAL of the right middle lobe).

a) Patlak-analysis derived influx constant (K_i)

Figure 5k illustrates how K_i and intercept are derived from a Patlak plot. MNC depletion had no significant effect on mean whole lung K_i (1.34 (SD 0.5) x10^{-3} ml blood ml lung^{-1} minute^{-1} in the sham sub-group and 1.43 (0.8) x10^{-3} ml blood ml lung^{-1} minute^{-1} in the depletion sub-group; P=0.78) (Figure 5k, Panel A). Mean K_i for left lung alone (i.e. excluding any potential BAL-induced inflammation within the right lung) was also unaffected by MNC depletion, with a mean of 1.26 (SD

0.6) x10^{-3} ml blood ml lung^{-1} minute^{-1} for the sham sub-group compared to 1.48 (1.0) x10^{-3} ml blood ml lung^{-1} minute^{-1} for the depletion sub-group (P=0.54) (Figure 5k, Panel B). Similarly, comparing a single lobe (left lower lobe) also demonstrated no significant difference in mean K_i between the two sub-groups (1.43 (SD 0.8) x10^{-3} ml blood ml lung^{-1} minute^{-1} compared to 1.74 (0.1) x10^{-3} ml blood ml lung^{-1} minute^{-1} in the sham and depletion sub-groups respectively; P=0.49).

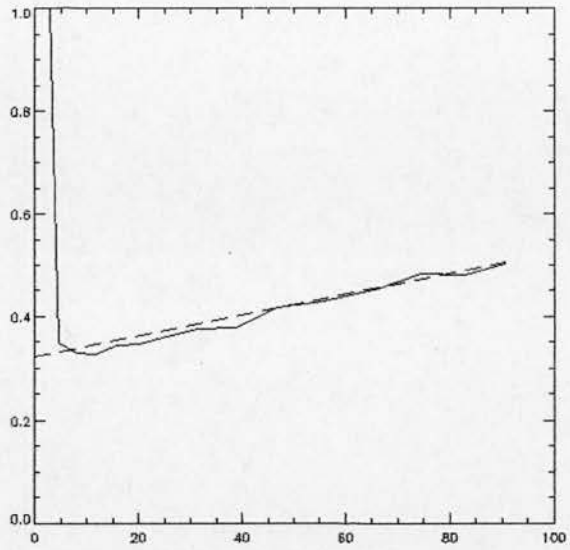


Figure 5j: Deriving influx constant (K_i) and intercept from a Patlak plot
The Patlak plot shown is an example from a subject in the sham group; the integrals of plasma [^{18}F]FDG activity divided by plasma [^{18}F]FDG activity are plotted on the x axis; the lung [^{18}F]FDG activity divided by plasma [^{18}F]FDG activity is plotted on the y axis; K_i is derived from the slope of the resultant line (where it becomes straight); the intercept of the straight line with the y axis can be used to adjust K_i for the volume of distribution of [^{18}F]FDG.

b) Intercept-adjusted K_i

MNC depletion did not significantly alter intercept-adjusted whole lung K_i (4.63 (SD 1.9) x10^{-3} minute^{-1} in the sham sub-group and 5.62 (3.9) x10^{-3} minute^{-1} in the depletion sub-group; P=0.48) (Figure 5k, Panel C). Mean intercept-adjusted

K_i for left lung alone also did not differ significantly between the sham and depletion subjects (4.16 (SD 2.1) $x10^{-3}$ $minute^{-1}$ compared to 5.99 (5.0) $x10^{-3}$ $minute^{-1}$ respectively; *P*=0.30) (Figure 5k, Panel D). Furthermore, MNC depletion had no significant effect upon mean intercept-adjusted K_i for left lower lobe (4.04 (SD 2.1) $x10^{-3}$ $minute^{-1}$ in the sham sub-group and 5.77 (4.4) $x10^{-3}$ $minute^{-1}$ in the depletion sub-group; *P*=0.27).

c) Standardised uptake value (SUV)

MNC depletion had no effect on mean whole lung SUV, which was 0.67 [IQR 0.58 – 0.78] in the sham sub-group and 0.67 [0.60 – 0.81] in the depletion sub-group (*P*=0.63) (Figure 5k, Panel E). Adjusting for the potential effect of BAL within the right lung did not alter the results, as mean SUV for left lung alone was also very similar between the groups (0.68 [IQR 0.57 – 0.79] and 0.66 [0.60 – 0.82] in the sham and depletion subjects respectively; *P*=0.80) (Figure 5k, Panel F).

d) Tissue-to-plasma activity ratio (TPR)

Mean whole lung TPR was also unaffected by MNC depletion (40.0 (SD 4.6) % in the sham sub-group and 39.7 (4.4) % in the depletion sub-group; *P*=0.86) (Figure 5k, Panel G). Mean TPR for left lung alone did also not differ significantly between the groups (39.9 (SD 5.5) % and 40.4 (5.0) % in the sham and depletion sub-groups respectively; *P*=0.85) (Figure 5k, Panel H).

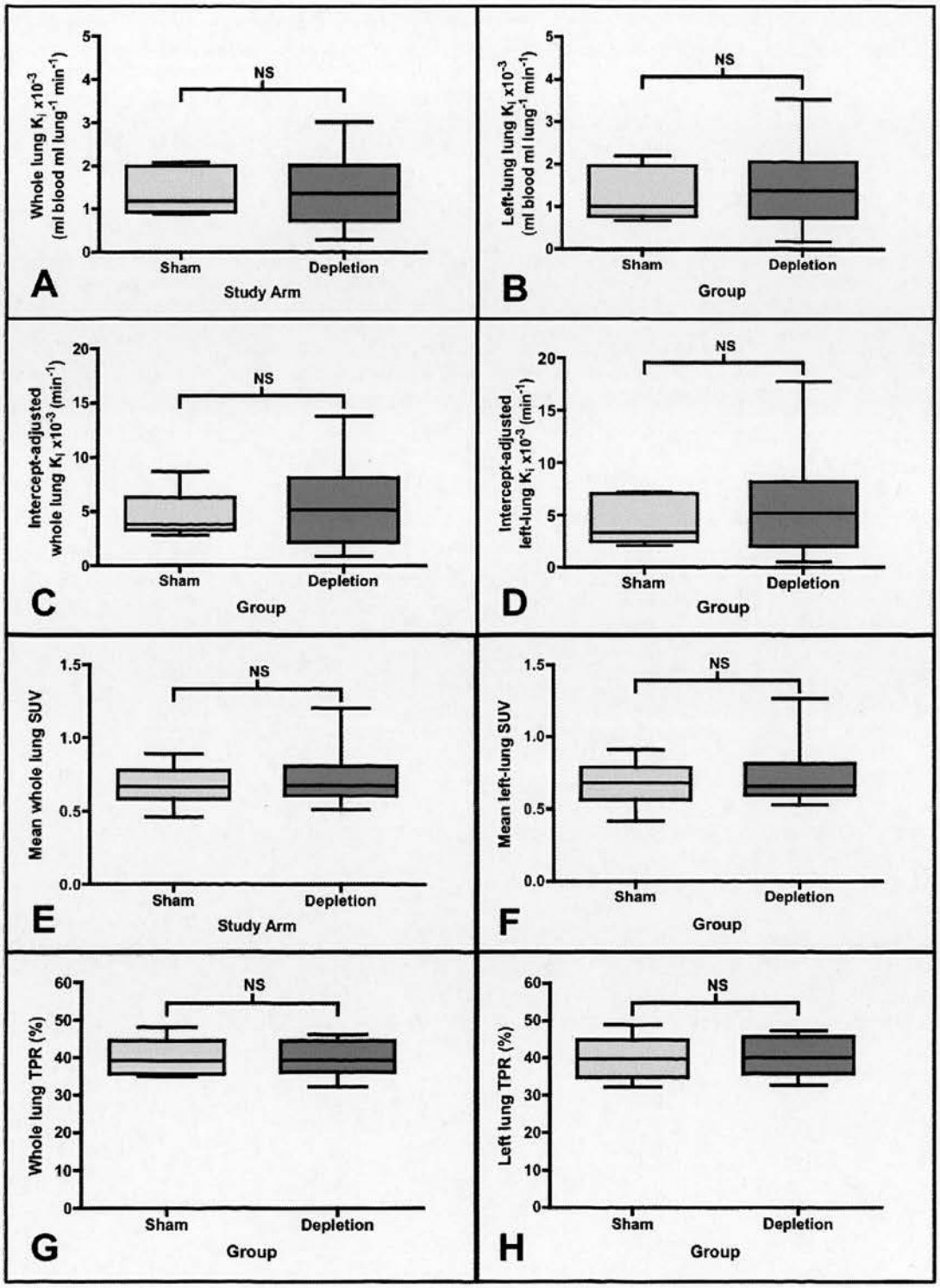


Figure 5k: Patlak analysis-derived K_i ([^{18}F]FDG uptake) in (A) whole lung and (B) left lung, intercept-adjusted K_i in (C) whole lung and (D) left lung, mean SUV in (E) whole lung and (F) left lung and TPR in (G) whole lung and (H) left lung

Data are presented as box (median/IQR) and whisker (range) plots; n=10 each group; statistical analysis was by 2-sample t-test or MWU test.

e) CT radiographic appearances

There was no clear difference between the groups in CT radiographic appearances. Three subjects from the sham group demonstrated inflammatory changes (nodular inflammatory changes in the left lower lobe in two subjects; nodular inflammatory change in the right lower lobe, with some ground glass opacification in the right middle lobe in the third subject). Four subjects in the depletion group had visible inflammatory changes on CT (ground-glass changes in the right middle lobe in three subjects, one of whom – together with the fourth subject – had nodular inflammatory change in the right lower lobe).

5.11 FURTHER ANALYSIS OF PMLC PHENOTYPE BY FLOW CYTOMETRY

The control (sham) group in Study C presented a valuable opportunity to further compare the phenotype of the resident and inducible PMLC subtypes present in BAL fluid after LPS inhalation, using flow cytometry.

The expression of several cell surface markers was first compared between the two PMLC subtypes in BAL fluid from the sham group subjects, who had inhaled LPS but who had not undergone MNC depletion (Table 5I). The iPMLC sub-type demonstrated significantly lower expression of the proliferation marker, Ki-67. The iPMLC population also demonstrated significantly lower expression of two markers of macrophage maturation: CD71 and CD206.

Expression of the macrophage scavenger receptor CD163 (a marker of maturation and anti-inflammatory activation) was very variable and did not differ significantly between the two PMLC subtypes.

Table 5I: Expression of cell surface markers of proliferation and macrophage maturation by the two PMLC subtypes in the sham group subjects

MARKER	rPMLC	iPMLC
Ki-67	19.0 [18.5 – 42.1]	2.1 [0.7 – 8.4] **
CD71	20.3 [15.7 – 31.7]	2.8 [1.5 – 3.6] ***
CD206	86.7 [72.8 – 97.9]	61.9 [40.6 – 73.9] *
CD163	92.2 [56.4 – 98.5]	69.6 [29.1 – 91.9] ^{NS}

Data are expressed as median [IQR] % of cells expressing each surface marker; n=12 (sham group only); statistical analysis was by MWU test; *P<0.05, **P<0.005, ***P<0.0005.

Expression of the same PMLC surface markers was also analysed in BAL fluid from the depletion group subjects: MNC depletion appeared to have no effect upon the differential patterns of Ki-67, CD71, CD206 and CD163 expression between the two PMLC subtypes.

5.12 FUNCTIONAL ANALYSIS OF FLOW-SORTED, CULTURED BAL CELLS

In addition to the phenotype data described above, some preliminary functional data relating to pro-inflammatory cytokine production were obtained by flow sorting and culturing BAL cell populations. The cell culture supernatants were extracted and levels of IL-6, IL-8 and TNFα were analysed using the CBA kits. Flow sorting was not performed in some subjects due to staff availability or

technical issues and flow sorting or cell culture were unsuccessful on some occasions, meaning that data are only complete for n=8 of the sham group. This sub-group did, however, remain representative of its parent group in terms of baseline characteristics.

There were insufficient cell numbers collected by flow sorting to obtain functional data for the rPMLC population. Functional data for flow sorted, cultured BAL cells from the depletion group were only available for a sub-group of n=6 and it was not therefore possible, with such a small sample number, to determine whether MNC depletion had any direct effect on BAL cell function in terms of pro-inflammatory cytokine release.

In the sham sub-group (where subjects had inhaled LPS but had not undergone MNC depletion), levels of spontaneous IL-6, IL-8 and TNFα production were very similar in the sorted iPMLC and AM populations. Levels of spontaneous pro-inflammatory cytokine production were compared to the levels recorded after dexamethasone suppression in the two cell types, per 10,000 cells cultured. IL-6 production demonstrated significant dexamethasone suppression in the AM population, compared to a much lesser (non-significant) extent in the iPMLC population (Figure 5l, Panels A and B). There was also significant dexamethasone suppression of IL-8 production by AMs, but not by iPMLCs (Figure 5l, Panels C and D). Finally, there was a non-significant trend towards dexamethasone suppression of TNFα production by the AM population, compared to no apparent effect in the iPMLC population (Figure 5l, Panels E and F).

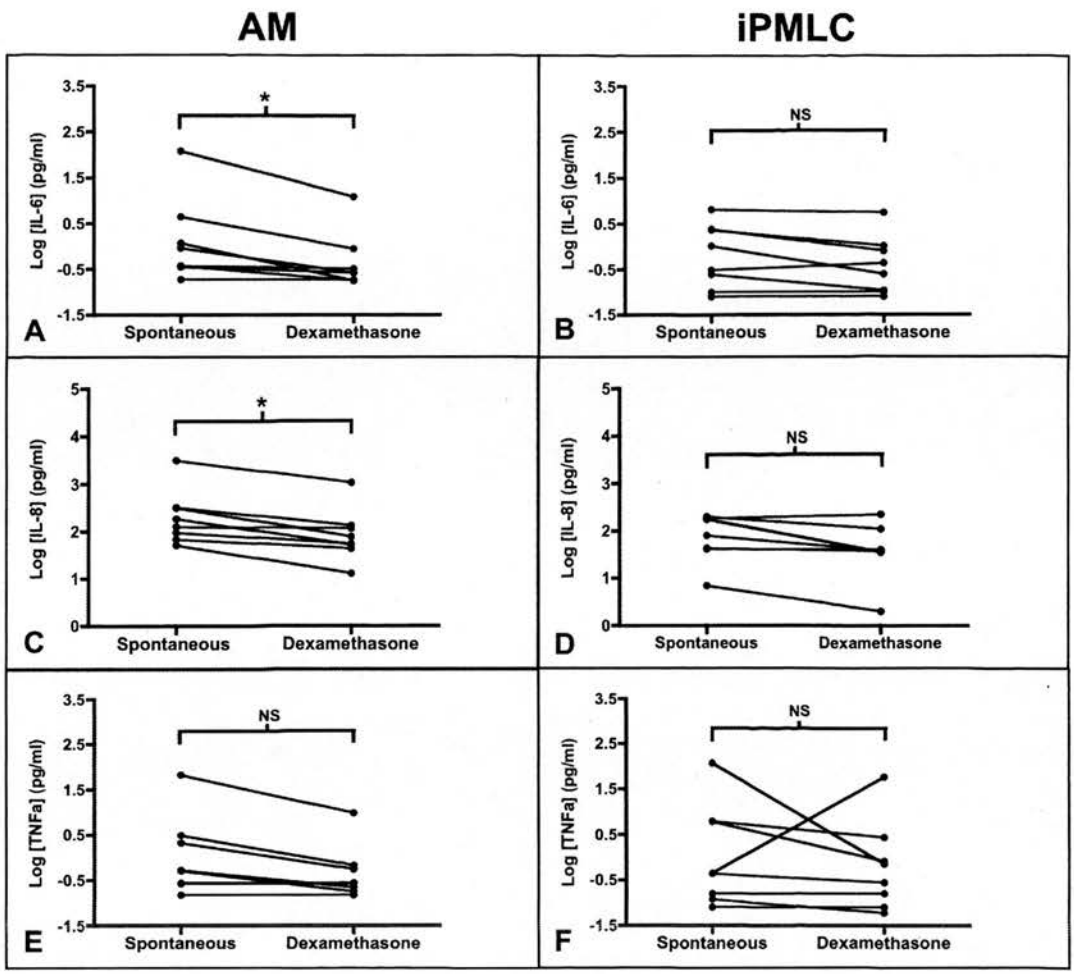


Figure 5l: Log concentrations of IL-6 (Panels A-B), IL-8 (Panels C-D) and TNFα (Panels E-F) in supernatants from flow-sorted, cultured AMs and iPMLCs

Data are shown paired (with/without incubation with dexamethasone) per 10,000 cells cultured; n=8 (sham group only); statistical analysis was by Wilcoxon signed rank test; *P<0.05.

5.13 CONCLUSIONS FROM STUDY C

a) Primary end-point

Mean circulating blood neutrophil count rose by 6.15 $x10^9$/L at 8 hours in the sham group subjects (i.e. similar to the rise of 5.2 $x10^9$/L seen in the Study A subjects who inhaled LPS). Contrary to my hypothesis, MNC depletion did not attenuate the LPS-induced rise in peripheral blood neutrophil count (the mean circulating neutrophil count rising by 6.16 $x10^9$/L in the depletion group subjects at 8 hours).

Small numbers of neutrophils were sequestered during leukapheresis in the depletion group subjects. For each subject, an adjusted 8-hour blood neutrophil count was calculated (to account for the number of neutrophils depleted by leukapheresis). This gave a mean rise in adjusted blood neutrophil count by 8 hours of 6.53 $x10^9$/L in the depletion subjects, with no significant difference compared to the sham group.

Furthermore, comparison of the overall pattern of LPS-induced change in blood neutrophil counts between baseline and 8 hours, incorporating the intermediate time-points of 2, 4 and 6 hours using a repeated-measures analysis, also demonstrated that MNC depletion had no significant effect.

b) Secondary end-points

Firstly, MNC depletion had no effect on mean SaO_2, with small and comparable falls noted in both the depletion and sham groups (these occurred around or

after the time of bronchoscopy, rather than as a clear direct effect of LPS inhalation).

Secondly, leukapheresis did not affect any of the measures of LPS-induced systemic inflammation measured in plasma. The rise in plasma CRP levels by 24 hours was not attenuated by MNC depletion. Similarly, the small rises in plasma IL-6 and IL-8 and the rise in plasma RAGE levels seen after LPS inhalation were not altered by leukapheresis.

Thirdly, MNC depletion had no effect on measures of LPS-induced alveolar inflammation, in particular BAL neutrophilia, which was around 30% in both groups. The higher levels of protein in BAL supernatant typically seen after LPS inhalation were not reduced by MNC leukapheresis. Furthermore, after LPS inhalation, the BAL supernatant levels of a variety of pro-inflammatory cytokines (IL-6 and IL-8), chemotactic cytokines (MCP-1 and MIP-1α), markers of neutrophil activation (HNE and MPO) and markers of cellular injury (RAGE and SP-D) were unaffected by MNC depletion.

Finally, the use of PET-CT in this study also allowed us to evaluate 'whole lung' inflammation, both in terms of anatomy (all lobes of both lungs) and pulmonary compartments (alveoli and interstitium) (Chen *et al.*, 2006; Jones *et al.*, 1997). MNC depletion did not influence any of the measures of LPS-induced pulmonary [^{18}F]FDG uptake. Values for K_i, intercept-adjusted K_i, mean SUV and TPR were similar in the sham and depletion sub-groups, even when BAL-induced changes

in [^{18}F]FDG uptake within the right lung were excluded by analysing data from left lung only.

c) Safety

There were no SAEs. As in the preliminary studies reported in Chapters 3 and 4, all reported symptoms or adverse events were attributable to LPS inhalation, leukapheresis/sham or other study procedures. As noted in the Study A LPS group, the majority of subjects in Study C reported pyrexial symptoms or developed a temperature >37.4 °C after LPS inhalation, but these symptoms were not sustained in any subject. Other symptoms attributable to LPS inhalation, including cough and headache, were also short-lived. Headache was a much more frequently reported symptom than in Study A, perhaps due to the combined effect of LPS inhalation followed by leukapheresis/sham.

Unlike in Study A, where FEV_1 readings all remained within 10% of baseline, there were four subjects in Study C (all from the sham group) whose FEV_1 fell by >10% below baseline. Although each subject reported associated cough or chest tightness, there was no audible wheeze on auscultation and these drops in FEV_1 resolved spontaneously without treatment in all four subjects. Overall, mean maximal % falls in FEV_1 and FVC were greater in Study C than in the Study A subjects who had inhaled LPS. Falls in FEV_1 and FVC similar to those seen in Study C (and after an equivalent dose of inhaled LPS) have been reported widely in previous literature (Kline *et al.*, 1999; Shyamsundar *et al.*, 2009; Thorn and Rylander, 1998).

Approximately half the subjects in Study C developed citrate-induced paraesthesiae during leukapheresis; all episodes resolved with IV calcium supplementation. This was very similar to Study B, with the relatively high frequency presumably due to the large volumes of blood processed. Importantly, there were no complications that could be attributed to infection in any of the subjects. Eight of the Study C subjects (n=4 from each group) reported light-headedness during or directly after leukapheresis and there was one vasovagal event, although the subject made a rapid recovery. This is in contrast to Study B, where there were no reports of light-headedness. This may be coincidence due to the smaller sample size; it is however possible that the effects of prior LPS inhalation might increase the frequency of light-headedness or vasovagal events. Vasovagal events are a well-recognised adverse event during leukapheresis, albeit usually only transient as was the case in this subject (Gasová *et al.*, 2010; Reik *et al.*, 1997; Strauss, 1996).

Study C thus confirmed that LPS inhalation and MNC leukapheresis are associated with a variety of predictable, short-lived side effects (as seen in the preliminary studies), but that they appear safe to use in healthy subjects.

d) Monocyte yield and subsets

As in Study B, a median volume of >20 litres of blood was processed during leukapheresis in both the sham and depletion groups. A mean of 7.7 $x10^9$ (median 7.3 $x10^9$) monocytes were retrieved in the MNC collections of subjects from the depletion group. This was higher than the median of 5.6 $x10^9$

monocytes retrieved during Study B, but the total number of circulating monocytes at baseline was also slightly higher (mean of 3.3 $x10^9$ compared to 2.9 $x10^9$ in Study B). There remained a very strong correlation between baseline circulating monocyte count and the number of monocytes retrieved by leukapheresis, as seen in Study B and previously reported in the literature (Nguyen *et al.*, 2002; Strasser *et al.*, 2003; Wolf *et al.*, 2005). Indeed, the monocyte yield in Study C was identical to that in Study B, at 2.3 (i.e. the number of monocytes collected during leukapheresis was more than twice the number circulating in blood at baseline).

e) Monocyte recruitment into circulating blood

I again found evidence of rapid recruitment of monocytes into the circulating blood pool during leukapheresis, as documented in Study B and by several other authors previously (Alteri and Leonard, 1983; Knudsen *et al.*, 2001; Nguyen *et al.*, 2002; Strasser *et al.*, 2005; Wolf *et al.*, 2005). Indeed, the depletion group subjects' mean circulating blood monocyte count fell to a much lesser extent than would be predicted from the number of monocytes removed during leukapheresis, indicating active monocyte recruitment into circulating blood during the procedure. In contrast to Study B, the mean circulating blood monocyte count in the depletion group fell by only 10% (compared to about 35% in Study B). This is almost certainly because LPS inhalation caused a rise in the mean circulating blood monocyte count by 8 hours, an effect that was clearly evident in the sham group subjects (where no monocytes had been removed by leukapheresis). This confirms the trend towards a rise in circulating blood

monocyte count that I had observed in the Study A subjects after LPS inhalation. In the Study C depletion group, the fall in circulating blood monocyte numbers (due to their depletion by leukapheresis) was therefore partially offset by this LPS-induced rise in blood monocyte counts. Both leukapheresis and LPS inhalation appear to cause monocyte recruitment, possibly from spleen and/or the marginating pool, into circulating blood.

f) Other effects of LPS inhalation

LPS inhalation was associated with marked rises in mean HR and mean temperature similar to those seen in the Study A subjects who had inhaled LPS. MNC depletion did not alter the LPS-induced rise in HR or temperature.

As noted in previous studies using LPS challenge (Michel *et al.*, 1995; O'Grady *et al.*, 2001), many of the cytokines and markers of cellular injury measured in plasma remained undetectable (for example, TNFα, IL-10, SP-D and vWF). LPS inhalation was, however, associated with significant rises in plasma markers of neutrophil activation and function (HNE and MPO), and to the same degree in the depletion group as the sham group. Interestingly, LPS inhalation appeared to result in a fall in the plasma levels of MCP-1 and SDF-1α (chemotactic cytokines) by 8 hours, a pattern not altered by MNC depletion. Previous studies have also commented upon chemokine down-regulation in inflammation (Fedyk *et al.*, 2001; Kim *et al.*, 2007). There was also an LPS-induced fall in circulating plasma sL-selectin levels in both groups that could potentially reflect selectin-

mediated neutrophil sequestration in the pulmonary vasculature (Kuebler *et al.*, 2000).

g) Pulmonary monocyte-like cells

There were no clear differences in flow cytometry plots of BAL cell size and granularity when comparing the two groups or when comparing these subjects with the LPS subjects from Study A. Compared to sham, MNC depletion was associated with a small fall in the proportion and estimated numbers of MNCs in BAL fluid, but had no effect upon the proportions of PMLC subsets. As seen in Study A after LPS inhalation, the large majority of PMLCs were of the 'inducible' subtype.

As discussed in Chapter 3 (Page 136), the dramatic rise in the proportion of iPMLCs present in BAL fluid after LPS inhalation may reflect transmigration of classical blood monocytes across the pulmonary endothelium. Further analysis of PMLC phenotype and function supports this theory.

Firstly, the iPMLC population demonstrated less dexamethasone-induced suppression of pro-inflammatory cytokine release when compared to AMs. Secondly, the weaker expression of macrophage maturation markers (CD71 and CD206) by the iPMLC subset suggests these cells may be less mature than the rPMLC subset. Furthermore, the rPMLC subset appeared to show greater proliferation potential than the iPMLCs, supporting the theory that this subset matures into alveolar macrophages or DCs (Gordon and Taylor, 2005; Landsman *et al.*, 2007; Landsman and Jung, 2007; Thomas *et al.*, 1976).

h) [^{18}F]FDG PET

As demonstrated above and discussed in Chapter 1, performing BAL provides the invaluable means to study the phenotype and function of inflammatory cells in the alveolar space. Although it does not provide equivalent information at a cellular level, [^{18}F]FDG PET has the advantage of measuring pulmonary inflammation within the interstitial as well as the alveolar compartment (including any cells adherent to the alveolar wall). Indeed, a study in rats given intratracheal LPS 18 hours earlier demonstrated that, within the lung, 94% of radiolabelled monocytes and 80% of radiolabelled neutrophils were located in the interstitium (as opposed to 6% and 20% respectively in BAL) (Li *et al.*, 1998).

Paired [^{18}F]FDG PET scans, before and after segmental LPS challenge, have previously been used to measure LPS-induced 'whole lung' inflammation within a single lobe by comparing subtraction to control images (Chen *et al.*, 2006; Chen *et al.*, 2009). In this study, I have shown for the first time that [^{18}F]FDG PET can also be used to quantify the more diffuse pulmonary inflammation produced by LPS inhalation, without the requirement for a baseline scan, and with no apparent interference by earlier BAL. Furthermore, the Ki values I obtained (of around 0.001 – 0.002 ml blood ml lung^{-1} minute^{-1}), are comparable with the segmental model and approximately ten times greater than control values (Chen *et al.*, 2009).

5.14 LIMITATIONS OF STUDY C

a) Sample size

This study was statistically powered to detect a significant reduction (of at least 2 x10^9/L) in the LPS-induced rise in circulating blood neutrophil count by 8 hours. This was a relatively small study in healthy subjects, using an experimental model of lung inflammation. As such, one of the limitations of this study is that it is difficult to predict the degree of reduction in blood neutrophil count that might translate to a clinically significant effect in patients with ALI.

A larger sample size may have also been useful to clarify any possible trends towards differences between the groups. The study may not have been sufficiently powered to detect subtle differences in, for example, the cytokines and cell injury markers that were measured.

PET-CT data was only obtained for a sub-group of twenty subjects, whereas ideally I would have performed PET-CT scans on all thirty subjects. Unfortunately, unavoidable delays and overall time constraint meant that study days had to proceed without PET-CT scans in ten subjects.

Similarly, functional data for iPMLCs and AMs in BAL were only available for a sub-group of 8 subjects from the sham group and this has to be taken into account when considering my results. I was also unable to accurately assess the effect of MNC depletion upon PMLC function.

b) Incomplete leukapheresis

I aimed to process 4 TBVs during leukapheresis (or sham) for each subject. In practice, some subjects had <4 TBVs processed (n=6 in depletion arm and n=5 in sham arm). Of these, the majority had ≥3.5 TBVs processed, with only 2 subjects from each arm having <3.5 TBVs processed. The reasons for incomplete (sham) leukapheresis were unavoidable and included the vasovagal event, venospasm or symptoms of citrate toxicity necessitating a lower flow rate.

c) Nature and efficacy of monocyte depletion

In Study B, analysis of the cellular content of three MNC collections indicated that leukapheresis may preferentially deplete the smaller, less dense, $CD16^+$ (intermediate and non-classical) monocyte subsets. This observation was supported by a rise in the proportion (equating to a smaller fall in estimated numbers) of classical monocytes in blood immediately post-leukapheresis in Study B. LPS inhalation, on the other hand, caused a rise in the estimated numbers of circulating classical monocytes, as shown in the LPS group from Study A and the sham group in Study C. Consistent with the combined effects of LPS inhalation followed by leukapheresis, estimated numbers of circulating classical monocytes remained static in the depletion group during Study C. I was unfortunately unable to analyse the monocyte content of the MNC collections in Study C (these were instead discarded), as this would have made it impossible to maintain blinding of the investigating team. Further work is therefore required to ascertain whether MNC leukapheresis preferentially removes $CD16^+$ monocyte subsets.

It could be argued that the small reduction in circulating monocyte numbers achieved by leukapheresis would not be sufficient to affect neutrophil influx or transmigration. Leukapheresis did, however, prevent the LPS-induced rise in circulating blood monocyte count that was seen in the sham group subjects. Furthermore, the depletion group subjects demonstrated a small reduction in the estimated numbers of MNCs in BAL fluid, suggesting that leukapheresis may have an impact, at least to some extent, upon LPS-induced pulmonary monocyte influx.

A longer duration of leukapheresis would have practical and safety implications. Fewer than four TBVs are processed during leukapheresis in standard clinical practice for collection of PBSCs. I chose to process four TBVs in order to maximise monocyte depletion within the available time, while prioritising subject safety and tolerance (keeping side effects, such as severe or prolonged citrate-induced paraesthesiae, to a minimum).

Although leukapheresis is readily available and safe for human use, methods of monocyte depletion employed in mice, including liposomal clodronate, anti-CCR2 monoclonal antibody and DT in CD11b-DTR transgenic animals, achieve much more effective and persistent monocyte depletion. Furthermore, methods of murine monocyte depletion affect other monocyte pools, for example bone marrow, splenic and patrolling monocytes, in addition to the circulating pool.

d) Depletion of other cells

As discussed in Chapter 4, MNC leukapheresis is not selective for monocytes and does deplete other cells, mainly lymphocytes, platelets and RBCs, but also (to a much lesser extent) neutrophils, basophils and eosinophils.

As in Study B, lymphocyte yield was >1 (indicating lymphocyte recruitment during the procedure) and strongly predictable from baseline circulating lymphocyte counts. The exact role of lymphocytes in ALI is unclear but T_{reg} cells may be key to the resolution of lung inflammation (D'alessio *et al.*, 2009). In Study A, I found a reduced proportion of T_{reg} cells in BAL fluid after LPS inhalation, although (as discussed in Chapter 3, Page 132) the absolute numbers of T_{reg} cells may have actually risen. An equivalent proportion of BAL T_{reg} cells was seen in both groups from Study C, appearing unaltered by MNC leukapheresis. Further work is required to establish whether absolute numbers of T_{reg} cells in BAL rise after LPS inhalation; the most accurate approach would probably involve the use of microbeads during flow cytometry to enable quantification of the T_{reg} population.

As expected, circulating blood lymphocyte counts fell significantly by 8 hours in the depletion group. Unexpectedly, they also fell (and to almost the same extent) in the sham group by 8 hours, raising the question of whether circulating lymphocytes were damaged by sham leukapheresis or somehow sequestrated in the centrifuge or tubing, although there are no previous reports of this in the literature. The much greater recovery in circulating blood lymphocyte counts by 24 hours in comparison to the depletion group is reassuring in this regard and

suggests more reversible reasons are responsible. Firstly, LPS inhalation may cause a fall in circulating blood lymphocyte counts; indeed, there was a trend towards this in the LPS subjects in Study A, although it did not reach significance. Secondly, the ACD-A anticoagulant infused during leukapheresis causes considerable expansion of circulating blood volume and a dilutional reduction in cell counts in the sham arm (as well as the depletion arm). Small falls in circulating blood platelet and RBC counts were also seen in the sham arm by 8 hours, almost certainly for this reason. Thirdly, circulating blood lymphocyte counts are known to vary on a diurnal basis, peaking around midnight and reaching a nadir around midday (Kronfol *et al.*, 1997; Lange *et al.*, 2010; Ritchie *et al.*, 1983; Sennels *et al.*, 2011), which may have contributed to my finding (the 8-hour time-point occurring in the early afternoon).

As in Study B, leukapheresis also depleted significant numbers of platelets, in proportion to circulating counts at baseline. Circulating blood platelet counts fell dramatically by 8 hours in the depletion group, but again to a lesser extent than would be predicted from the platelet yield of 0.8 (indicating active recruitment during the procedure). Evidence from murine models indicates that platelets also play a key role in the pathogenesis of ALI (Caudrillier *et al.*, 2012; Grommes *et al.*, 2012; Kornerup *et al.*, 2010; Looney *et al.*, 2009; Zarbock and Ley, 2009); retrospective studies of critically ill patients have also shown that platelet transfusions increase the risk of developing ALI (Khan *et al.*, 2007) and that aspirin therapy may reduce the risk (Erlich, 2011; Kor *et al.*, 2011); a multi-

centre RCT of aspirin therapy for the prevention of ALI is currently underway (Kor *et al.*, 2012).

A large number of RBCs were retrieved into the MNC collections from leukapheresis. As in Study B, however, this represented only a very small proportion (0.7%) of total circulating RBCs at baseline. Transfusion of RBCs has been noted to predispose to the development of ALI and increase subsequent mortality in a dose-dependent manner (Gong *et al.*, 2005). Duffy antigen (expressed on RBCs) has been shown to scavenge pro-inflammatory chemokines and prevent excessive neutrophil transmigration into the alveolar space in a murine model of ALI (J S Lee *et al.*, 2006; Reutershan *et al.*, 2009). Red cell storage is associated with loss of Duffy antigen expression (Mangalmurti *et al.*, 2008). Furthermore, oxidised haemoglobin has pro-inflammatory actions, including disruption of the endothelial barrier.

It is therefore possible that the depletion of lymphocytes, platelets and RBCs by leukapheresis could have had an impact upon LPS-induced inflammation in this study. It does seem likely, however, that their depletion would if anything accentuate rather than mask any beneficial effect of monocyte depletion.

Neutrophils were sequestered from 9 out of the 15 depletion group subjects during MNC leukapheresis. Although neutrophil depletion occurred more frequently than expected compared to Study B (where only 1 out of the 6 subjects demonstrated neutrophil depletion by leukapheresis), its extent was limited, representing on average only 0.05 times the number of neutrophils

circulating in blood at baseline. The increased frequency of neutrophil depletion is probably partly due to the LPS-induced rise in blood neutrophil counts.

Another potential reason for neutrophil contamination of MNC collections is usually due to fluctuations in venous flow during leukapheresis, which was noticeable in several of the subjects and probably the main explanation for neutrophil sequestration. Venospasm disrupts the interface by altering the height of the cell sediment, thus requiring the operator to perform frequent readjustments to maintain the collect line haematocrit between 1-3% (to optimise MNC collection and minimise neutrophil contamination) (Feige and Sorg, 1984; Linenberger, 2005a; Strasser and Eckstein, 2010). Reducing the cell collection rate or using online sampling during leukapheresis (to improve adjustment of the interface) can both help to minimise neutrophil contamination, but have practical implications, particularly in terms of procedure duration and consequent tolerability (Faradji *et al.*, 1994; Svensson *et al.*, 2005).

e) Effects of sham leukapheresis

Sham leukapheresis is not a true 'placebo', as it involves extra-corporeal blood diversion, anticoagulation and centrifugation with resultant cell sedimentation. Although whole blood is then returned into the circulation (without any cell collection), it is possible that sham leukapheresis may cause cellular activation or have other immune-modulating effects. As discussed in Chapter 4, sham leukapheresis does not appear to affect monocyte chemotaxis (Alteri and

Leonard, 1983) but other literature on this topic is scant. When compared to the Study A subjects who had inhaled LPS (but who did not undergo sham leukapheresis), there was a slightly higher rise in mean blood neutrophil count (6.15×10^9/L versus 5.24×10^9/L), higher mean total BAL cell count (4.5×10^5/ml versus 3.1×10^5/ml), higher mean BAL protein content (0.24 g/L versus 0.15 g/L) and higher mean maximum rise in temperature (1.9 °C compared to 1.4 °C), that could represent augmentation of LPS-induced inflammation by sham leukapheresis. Conversely, the sham group subjects demonstrated a similar mean total number of BAL neutrophils (160×10^3/ml versus 172×10^3/ml) and similar median BAL IL-8 concentrations (61 pg/ml compared to 79 pg/ml).

If it does have unintended effects, sham leukapheresis is most likely to potentiate the inflammatory effects of LPS inhalation in the sham group, rather than conceal any beneficial effect of monocyte depletion in the intervention group. Sham leukapheresis had a distinct advantage over using a control group with no leukapheresis, as it enabled double blinding of my study, so that both subjects and the investigating team were unaware of randomisation status, thus avoiding any associated bias.

f) Limitations of the LPS model

The limitations of the LPS model were discussed at the end of Chapter 3 (Page 143). Very similar concentrations of LPS were detectable in BAL fluid samples from all subjects in Study C. Furthermore, PET-derived measures of global lung inflammation were not affected by analysing data from the left lung alone, or

even the left lower lobe alone, compared to both lungs. These findings suggest reasonably even and consistent distribution of inhaled LPS by this method.

g) Timing of end-points and intervention

The timing of the primary end-point was chosen because the rise in blood neutrophil count after LPS inhalation tends to peak at around 8 hours. This time point coincided with the end of leukapheresis and, crucially, preceded BAL (which can itself influence blood neutrophil counts, as noted in the preliminary studies). It is possible that the timing of the primary end-point was too early to detect an effect of MNC depletion, but no significant differences were seen in either of the two secondary end-points measured on day 2 (plasma CRP levels and PET-derived K_i and mean SUV).

Leukapheresis temporarily caused a reduction in the circulating monocyte pool, with no improvement in various measures of LPS-induced inflammation. This may be because leukapheresis did not influence other monocyte pools. Firstly, for example, there is evidence that patrolling monocytes (present in the pulmonary vasculature) play a key role in early neutrophil recruitment to the lung in ALI (Auffray *et al.*, 2007). Secondly, LPS inhalation may induce splenic monocyte release, as has been shown in other models of inflammation (Swirski *et al.*, 2009). Leukapheresis may then augment any mobilisation of the splenic monocyte population, accounting for the rapid and striking repopulation of the circulating monocyte pool during leukapheresis.

5.15 SUMMARY OF STUDY C

This RCT was designed to build upon murine studies, where monocyte depletion has been shown to substantially reduce LPS-induced lung inflammation, to test whether this finding can be extrapolated to humans. I have shown that, contrary to my hypothesis, MNC depletion by leukapheresis does not attenuate LPS-induced systemic or pulmonary inflammation in healthy human subjects.

It does, however, remain likely that monocytes play a significant role in the complex pathogenesis of ALI. The final chapter of my thesis outlines the need for more human studies, both to further define the exact role of monocytes in human lung inflammation and to investigate other potential methods of modulating monocyte migration and/or function in lung inflammation, with the aim of identifying new therapeutic options for patients with ALI.

CHAPTER 6: FUTURE WORK

6.1 METHODOLOGICAL CONSIDERATIONS

Firstly, leading on from my earlier discussion on the limitations of all three studies, I shall reflect on how using alternative or additional controls may have improved my study design.

In relation to Study A, a randomised crossover-type study design (incorporating a suitable washout period between inhalation challenges) would have enabled a within-subject comparison of LPS and saline inhalation. This would, however, necessitate two separate study days and therefore significantly increase the risk of subject withdrawal mid-way through the study. It would have been interesting to perform PET-CT scans in the Study A subjects, to compare the differences in PET-derived measures of global lung inflammation after LPS inhalation when compared to saline inhalation.

In retrospect, it would have been extremely useful to examine the effects of sham leukapheresis in a control group within Study B (i.e. without the potentially confounding effects of LPS inhalation as were present in Study C). This could have utilised a randomised double-blind design, with six subjects undergoing sham leukapheresis as well as six subjects undergoing true leukapheresis and MNC collection. Alternatively, Study B could also have been performed using a crossover design with suitable washout period, allowing comparison of true and sham leukapheresis within each subject. As discussed previously, sham leukapheresis may have subtle modulating effects on systemic

and pulmonary inflammation. Close scrutiny of the effects of sham leukapheresis to qualify its use as a 'negative control' would have been useful prior to proceeding with the RCT.

Within Study C, as stated in Chapter 5, it would have been preferable to perform PET-CT scans in all thirty subjects had time constraints allowed. An alternative study design would have involved four-way randomisation, firstly to either LPS or saline inhalation and then to either MNC depletion or sham leukapheresis. Such an approach may have enabled more precise comparisons of the effects of MNC depletion and sham leukapheresis and also the effects of LPS compared to saline inhalation, specifically with regards to PET-derived measures of global lung inflammation. The use of PET-CT scanning limits the use of crossover studies in healthy volunteers due to the need to avoid repeat doses of radiation.

A further possible methodological alteration within Study C would have involved employing a second control group of healthy subjects who also inhaled LPS but who did not undergo sham leukapheresis. Although this additional group's treatment could clearly not be blinded, it would have provided a useful means to compare the effects of sham leukapheresis with no leukapheresis (and with MNC collection).

6.2 FUTURE WORK

a) The role of monocytes in lung inflammation

As discussed in Chapter 1, the pathophysiology of ALI is extremely complex and translation of findings from animal studies into pharmacological treatments for ALI has proved problematic. There is, at least in mice, substantial and wide-ranging evidence suggesting that monocytes play an important role in the pulmonary neutrophil transmigration that typically occurs as ALI evolves (Dhaliwal *et al.*, 2012; Ebner *et al.*, 2010; Henderson, 2003; Janardhan *et al.*, 2006; Maus *et al.*, 2001).

More work is needed to elucidate the exact role of monocytes in human lung inflammation and thus to guide potential future interventions. Very little is known about pulmonary monocyte influx, subsequent maturation and cell phenotype, as is illustrated by the varying descriptions of 'monocytes', 'monocyte-like cells' and 'small macrophages' in BAL or sputum from patients with lung inflammation. As discussed in earlier chapters, cellular identification by light microscopy is not straightforward. With the studies described in this thesis, I have shown that flow cytometry can be employed to define and further characterise these cells (PMLCs) according to their cell surface marker expression. Furthermore, LPS-induced pulmonary inflammation appears to be accompanied by an influx of iPMLCs, the PMLC subtype that resembles classical monocytes in circulating blood, by 9 hours.

In terms of further work, it would firstly be interesting to study BAL at other (both earlier and later) time-points after LPS inhalation to more precisely define changes in PMLC populations over time. It could also potentially be useful to compare PMLC populations in BAL in the same subject before and after LPS inhalation (thus providing a within-subject control) although this would necessitate two bronchoscopy procedures per subject and a suitable 'washout' period after initial BAL.

The second step in future work would be to use flow cytometry to clarify whether equivalent populations of PMLCs are present in BAL from patients with ALI (and other inflammatory lung conditions) and whether these correspond to the 'alveolar monocytes' described by Rosseau *et al* (2000). It would also be useful to study the kinetics of PMLC populations (especially in relation to neutrophil populations and other markers of alveolar inflammation) at varying time-points in patients with ALI. In addition to flow cytometry, it would also then be interesting to perform further functional studies of flow-sorted PMLC populations, ideally obtaining sufficient numbers of rPMLCs to enable direct comparisons between the two PMLC subtypes, and also with AMs and neutrophils.

With respect to circulating monocytes in blood, my findings from flow cytometry support recent evidence for three human monocyte subsets distinguishable by their cell surface marker expression. My results also indicate a rise in the numbers of circulating classical monocytes in blood after LPS inhalation. The next challenge is to demonstrate whether the apparent influx of

$CD14^{++}CD16^{-}$ iPMLC present in BAL after LPS inhalation is due to the accompanying rise in circulating $CD14^{++}CD16^{-}$ (classical) monocytes and their subsequent pulmonary transmigration. ^{1}H MRI represents an expanding non-invasive imaging modality that does not necessitate the use of radioactive tracers. One possibility for tracking and imaging circulating monocytes is to label them (ex-vivo) with superparamagnetic iron oxide (SPIO) particles. Reassuringly, there is no evidence that this alters monocyte function or migration and MRI can then be used to track monocyte migration to sites of inflammation (Richards *et al.*, 2012). SPIO particles do, however, reduce MR signal and this therefore limits their use in low-density lung tissue. An alternative is to intravenously administer ^{19}F perfluorocarbon nanoparticles, which are then phagocytosed by circulating monocytes. These are biochemically inert, have a good safety profile for clinical use and result in more persistent cellular labelling detectable by MRI scanning (Ebner *et al.*, 2010; Flögel *et al.*, 2008; Srinivas *et al.*, 2012).

Assuming monocytes do play an exacerbating role in neutrophilic lung inflammation, there are a number of potential reasons (as discussed in Chapter 5) why monocyte depletion by leukapheresis proved ineffective in Study C. Potential methodological adjustments and alternative strategies are discussed below.

b) Timing of monocyte depletion

In this study, leukapheresis was designed to begin 2 hours after LPS inhalation and the effect on circulating blood monocyte counts only therefore became noticeable at around 4 hours from baseline. As discussed in Chapter 1, there is evidence of pro-inflammatory cytokine release and both a rise in BAL cell numbers and blood neutrophils within 4 hours of LPS inhalation. It may be that earlier leukapheresis would prove to be a more effective strategy, although this may at the same time make it less clinically translatable, as it is difficult to identify patients with ALI at an early stage. A pre-emptive approach might prove more practical, but would limit the clinical applicability to patients at high risk of ALI, rather than those with established lung inflammation.

In mice (albeit in a peritoneal model of inflammation), patrolling resident monocytes have been reported to extravasate within 1 hour of tissue damage (Auffray *et al.*, 2007). Further research is required to determine whether early extravasation of patrolling monocytes in pulmonary endothelium is important in the pathogenesis of ALI. Interestingly, in humans, rapid and high-volume mobilisation of 'marginating' $CD14^+$ $CD16^+$ monocytes into the circulating pool has been described after strenuous exercise (Steppich *et al.*, 2000). As I have shown in Studies B and C, removing circulating monocytes by leukapheresis induces rapid monocyte recruitment, presumably from other monocyte pools. It is therefore feasible that the pre-emptive use of leukapheresis could interfere with the pathogenesis of lung inflammation by recruiting patrolling monocytes into the circulation and thus preventing their extravasation.

Alternatively, it is possible that I missed a later effect of leukapheresis; although I did not measure BAL at later time-points, there was no indication that leukapheresis had a significant effect upon other markers of systemic or pulmonary inflammation that were measured at/after 24 hours (including plasma CRP levels and [^{18}F]FDG PET-derived K_i). In the same vein, it could also be argued that I timed leukapheresis to occur too early after the initiation of LPS-induced lung inflammation. It is possible that chemokine release stimulates the initial neutrophil transmigration that occurs in ALI and that monocytes are only involved in the second, later phase of pulmonary neutrophil influx that peaks at 24-48 hours (Dhaliwal *et al.*, 2012; Petty *et al.*, 2007).

c) Nature of monocyte depletion

Safety is one of the most important considerations when translating research into clinical practice (especially when studying healthy volunteers). This is particularly relevant to ALI, where the normal, protective host response to an injurious stimulus appears to evolve into excessive and uncontrolled inflammation; clearly any intervention that compromises the protective nature of the host response could ultimately prove harmful. As discussed earlier, leukapheresis had the advantage of being a readily available technique, with some evidence of effect in other inflammatory conditions and, crucially, a good safety profile. However, compared to the methods of monocyte depletion that have been described in mice, it provided a relatively limited, transient and non-selective effect. Research is therefore needed into other potential methods of

human monocyte depletion that may have a more profound, sustained and selective effect than leukapheresis.

One possible area for such research includes the use of monoclonal antibodies that interfere with monocyte function or migration. The use of monoclonal antibodies represents a rapidly expanding field, with a growing list of applications including autoimmune inflammatory conditions, viral diseases, transplant rejection, cancer, asthma and stroke (Hansel *et al.*, 2010; Hudson and Souriau, 2003). For example, with relevance to monocyte chemotaxis, one group tested whether an anti-MCP-1 monoclonal antibody could reduce disease severity in patients with RA (Haringman *et al.*, 2006). Following administration, they noted a quick fall in circulating free levels of MCP-1 in blood, but demonstrated no clinical or histological improvement. Indeed, at the highest dose, they noted a possible trend towards increased inflammation; the authors attributed this paradoxical finding to an unexpected, steep rise in circulating levels of antibody-bound MCP-1 in blood (hypothesised to reflect reduced antibody clearance) (Haringman *et al.*, 2006).

Another group, this time in mice, reported that using a monoclonal antibody to block junctional adhesion molecule-C receptors reduced net monocyte influx to inflammatory tissues (Bradfield *et al.*, 2007). These authors argued that higher circulating levels of monocytes that had lost CD62L expression reflected increased monocyte reverse transmigration across the endothelium (Bradfield *et al.*, 2007).

There are many potential targets for monoclonal antibody therapy in patients with ALI; however, safety is again a key consideration. A variety of potentially serious or even fatal adverse reactions to monoclonal antibody therapy have been reported, including immune reactions such as anaphylaxis and serum sickness, infections, cancer, autoimmune disease and cytokine release syndrome (Hansel *et al.*, 2010). For example, severe cytokine release syndrome occurred in six healthy volunteers given a superagonist anti-CD28 monoclonal antibody in a now notorious phase I human trial in 2006 (Suntharalingam *et al.*, 2006). This has lead to a more cautious approach to deciding the initial dose for biological agents being used in humans for the first time (Hansel *et al.*, 2010).

Another possible area for research relates to the contribution of other monocyte pools. The difficulty in achieving a sustained effect from leukapheresis was partly due to time constraints relating to tolerability but also due to marked recruitment of monocytes into circulating blood during the procedure that may have negated the intended effect. Indeed, in monocyte-depleted mice given intratracheal LPS, Dhaliwal *et al* (2012) showed that adoptive transfer of freshly isolated MNCs lead to a prompt rise in BAL neutrophil numbers. Extensive work in mice has demonstrated the presence of a splenic pool of monocytes and it is certainly possible that splenic monocyte depletion may have partly contributed to the attenuation of LPS-induced lung injury in previous murine studies.

Research done in mice and rats employing coronary ligation to induce myocardial infarction demonstrated a reduction in the splenic monocyte pool (compared to no reduction in monocyte numbers present in bone marrow), a

rise in both subtypes of circulating monocytes and very high Ly6C^{high} monocyte content in ischaemic myocardium (Swirski *et al.*, 2009). Splenectomy prevented the rise in circulating blood monocytes and reduced the numbers of Ly6C^{high} monocytes in ischaemic myocardium by 75% (Swirski *et al.*, 2009). Swirski *et al* also provided evidence that monocytes were released from the spleen under the control of angiotensin II and that these monocytes are biologically active. This is in contrast to CCR2-dependent monocyte exit from bone marrow (Serbina and Pamer, 2006). Another paper confirmed the early accumulation of Ly6C^{high} monocytes in ischaemic myocardium, compared to a later rise in Ly6C^{low} monocytes that peaked at about 7 days (Nahrendorf *et al.*, 2007). Ongoing splenic monopoiesis appears to occur in both the myocardial infarction and a cerebral infarction model (Leuschner *et al.*, 2012). This group have demonstrated, using time-lapse intravital fluorescence microscopy in GFP knock-in mice, that inhibition of angiotensin converting enzyme (ACE) prevented the release of splenic monocytes after coronary ligation (Leuschner *et al.*, 2010). Furthermore, ACE inhibition attenuated the rise in circulating monocytes and also their accumulation in ischaemic myocardium, in particular the early peak in Ly6C^{high} monocytes (Leuschner *et al.*, 2010). A similar but less marked effect was achieved by splenectomy.

It would be extremely interesting to study splenic monocyte kinetics in mice with experimental ALI, especially as the renin-angiotensin system has been implicated in neutrophil influx in animal models of lung inflammation. For example, using both a sepsis and acid aspiration model, one group showed that

lung injury was less severe in mice deficient in ACE; furthermore recombinant ACE2 (which blocks the angiotensin II receptor) protected mice against lung injury (Imai *et al.*, 2005). Arndt *et al* (2006) went on to demonstrate a significant reduction in pulmonary neutrophil recruitment after LPS in mice pre-treated with either an ACE inhibitor (enalapril) or an angiotensin II receptor blocker (losartan).

d) Summary

In conclusion, the priorities for further research in this field include firstly establishing whether PMLC populations are present in BAL fluid from patients with ALI, secondly using imaging to establish monocyte kinetics after inhaled LPS in healthy volunteers and, thirdly, confirming whether splenic monocyte release is relevant to animal models of lung injury.

If an equivalent population of splenic monocytes can be confirmed in humans, then targeting their release using ACE inhibition or blockade of the angiotensin II receptor would appear to be the most promising area for further translational research. This would also provide an excellent candidate treatment (with a good, established safety profile) for a further phase II study employing the LPS inhalation model in healthy human subjects.

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PUBLICATIONS ARISING FROM THIS WORK

Study A (Appendix 1)

Brittan M., Barr L. Conway Morris A., Duffin R., Rossi F., Johnston S., Monro G., Anderson N., Rossi G., McAuley D.F., Haslett C., Hirani N., Dhaliwal K. and Simpson A.J. (2012). A novel subpopulation of monocyte-like cells in the human lung after lipopolysaccharide inhalation. *European Respiratory Journal* **40**: 206-14.

Study B (Appendix 2)

Barr L., Brittan M., Conway Morris A., Stewart A., Dhaliwal K., Anderson N., Turner M., Manson L. and Simpson A.J. (2012). Pulmonary and systemic effects of mononuclear leukapheresis. *Vox Sanguinis* **103**: 275-83.

Study C (Appendix 3)

Barr L.C., Brittan M., Conway Morris A., McAuley D.F., McCormack C., Fletcher A.M., Richardson H., Connell M., Patel D., Wallace W.A.H., Rossi A.G., Davidson D.J., Manson L., Turner M., Hirani N., Walsh T.S., Anderson N.H., Dhaliwal K. and Simpson A.J. (2013). A randomized controlled trial of peripheral blood mononuclear cell depletion in experimental human lung inflammation. *American Journal of Respiratory and Critical Care Medicine* **188** (4): 449-55.